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A thesis submitted for the degree of Doctor of Philosophy

Multimodal biomarkers to improve the diagnosis and treatment of autonomic diseases

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Declaration

I, Shiwen Koay, confirm that the work presented in my thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated and referenced in the thesis.

Abstract

The autonomic nervous system orchestrates the vital but unconscious control of every organ in the body. Autonomic failure can be a primary feature in several autoimmune and neurodegenerative diseases, presenting with severe disabling orthostatic hypotension, genitourinary and pupillary deficits. Overlapping clinical features at initial presentation may lead to diagnostic uncertainty. We explored whether multiple physiological and morphological biomarkers could identify characteristic phenotypes to distinguish between different autonomic diseases and quantify response to treatment.

Firstly, we demonstrated ganglionic acetylcholine receptor (gAChR-positive) patients with autoimmune autonomic ganglionopathy (AAG) had a distinct phenotype with severe widespread parasympathetic and sympathetic autonomic failure, with prominent cholinergic deficits, and defined biomarkers to quantify improvements following immunotherapy. In contrast, gAChR-negative patients had heterogenous phenotypes, with a subset demonstrating sympathetic predominant autonomic failure, with significantly greater cutaneous adrenergic denervation, suggesting a different pathophysiological process.

We then described the largest longitudinal cohort to date of patients with neurodegenerative α-synucleinopathies, including pure autonomic failure (PAF), multiple system atrophy (MSA), and Lewy body diseases (LBD), including Parkinson's disease (PD) and Dementia with Lewy bodies (DLB). Patients with PAF had significantly greater orthostatic hypotension, lower supine noradrenaline, and more frequent sympathetic pupillary deficits, suggesting greater postganglionic adrenergic denervation compared to other patients. Normal pupils, supine noradrenaline, and less severe orthostatic

hypotension at initial assessment predicted conversion to a more widespread α-synucleinopathy by final assessment.

We applied indirect immunofluorescence techniques to assess for cutaneous neural phosphorylated synuclein (p-syn) deposits in PAF, MSA, and PD, using a semi-quantitative score to describe the presence of p-syn on autonomic and other cutaneous nerves. Total p-syn scores were significantly higher in PAF compared to MSA and PD, consistent with a peripherally predominant α-synucleinopathy. Autonomic p-syn subscores correlated with severity of cardiovascular autonomic failure, suggesting p-syn deposition on autonomic nerves may contribute to the pathophysiology of autonomic failure.

Impact statement

The Autonomic Unit at the National Hospital for Neurology and Neurosurgery was established in 1975. In the last fifty years, it has developed from a small testing facility into the largest autonomic referral centre in Europe, providing comprehensive autonomic testing and expert multi-disciplinary advice to clinicians across the UK and Europe on the diagnosis and management of patients with autonomic failure. This exceptional environment has enabled us to produce high quality research to deepen our understanding and improve the management of these disabling but potentially treatable diseases.

This research project was driven by the need to find early objective biomarkers to improve the diagnosis and treatment of patients with autonomic failure. The studies presented in this thesis include the largest and most comprehensively phenotyped

longitudinal cohorts worldwide of patients with rare autoimmune and neurodegenerative autonomic diseases seen at a single autonomic referral centre over the last twenty years. Through deep phenotyping with autonomic and morphological biomarkers, we have gained insights into the pathophysiology and natural history of these diseases, describing novel diagnostic and prognostic biomarkers, as well as strategies for treatment and monitoring response to therapy.[1] The research has already had impact on clinical practice at our centre, nationally and internationally, with increased calls for the use of multimodal objective biomarkers in the management of patients with autonomic diseases.[2-5] The NHS England Immunoglobulin Expert Working Group have recently updated their commissioning criteria to include autoimmune autonomic ganglionopathy as an indication for routine commissioning, with guidance on supportive clinical features, exclusion criteria, treatment strategies and outcome measures, incorporating results from our research (Appendix 1 and 2).

A clinically important challenge is differentiating between patients with autoimmune and neurodegenerative autonomic diseases, especially in patients with atypical features causing diagnostic uncertainty. We initially laid out clinical features and autonomic biomarkers that could help to distinguish between autoimmune and degenerative diseases and have now identified cutaneous neural phosphorylated synuclein deposits as an extremely promising pathological biomarker which appears to perfectly separate patients with autoimmune and neurodegenerative pathologies. This will allow clinicians to make more accurate diagnoses at an earlier stage of the disease and potentially avoid trials of unnecessary immunotherapy in patients with neurodegenerative diseases, with financial and clinical implications. Improved diagnostic biomarkers will facilitate earlier

recruitment to future clinical trials, when novel disease modifying therapies are more likely to have an impact.

We now have a cohort of prospectively recruited, deeply phenotyped patients with multimodal autonomic biomarkers, blood samples, and skin biopsies, which represent an invaluable resource for further research, with ongoing and future studies planned. We have uncovered exciting insights into the pathophysiology and potential recovery and regeneration in patients with autoimmune autonomic failure, with novel biomarkers that could be adapted to study other related diseases. Through national and international collaborations, we have built a thriving portfolio of clinically relevant research, establishing the Autonomic Unit as one of the world leaders in the field.

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Contents

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Abbreviations

AAG	Autoimmune autonomic ganglionopathy
CASS	Composite Autonomic Severity Score
COLIV	Collagen IV
COMPASS	Abbreviated and refined composite autonomic symptom score
DβH	Dopamine-beta-hydroxylase
DLB	Dementia with Lewy Bodies
DPX	Dibutylphthalate polystyrene xylene
DST	Dynamic sweat testing
gAChR	Ganglionic acetylcholine receptor
GBS	Guillain-barre syndrome
GFAP	Glial fibrillary acidic protein
HR_{DB}	Heart rate variability with deep breathing
IENF	Intraepidermal nerve fibres
IVIg	Intravenous immunoglobulin
LBD	Lewy Body diseases
MBP	Myelin basic protein
MIBG	^{123}I-meta-iodobenzylguanidine

MSA	Multiple system atrophy
MSA-P	Multiple system atrophy-Parkinsonian subtype
NA	Noradrenaline
OH	Orthostatic hypotension
OIR-stand	Orthostatic intolerance ratio on stand
OIR-tilt	Orthostatic intolerance ratio with head-up tilt
PAF	Pure autonomic failure
PBS	Phosphate buffered sucrose
PD	Parkinson’s disease
PGP	Protein gene product 9.5
PET	Positron emission tomography
PRT	Pressure recovery time
P-syn	Phosphorylated-synuclein
QSART	Quantitative sudomotor axon reflex test
RBD	Rapid eye movement sleep Behaviour Disorder
REM	Rapid eye movement
SBP	Systolic blood pressure
SF-36	36-item short form health survey

SFN-SIQ	Small fibre neuropathy symptom inventory questionnaire
SPECT	Single-photon emission computerised tomography
VIP	Vasoactive intestinal peptide
VR	Valsalva ratioF

Chapter 1. Introduction

1.1 The autonomic nervous system in health and disease

The autonomic nervous system is responsible for the unconscious but vital control of all organs in the body. A complex neural network innervates all organs via complementary thoracolumbar sympathetic and craniosacral parasympathetic pathways (Figure 1.2), with specific neurotransmitters that influence ganglionic and post-ganglionic function.

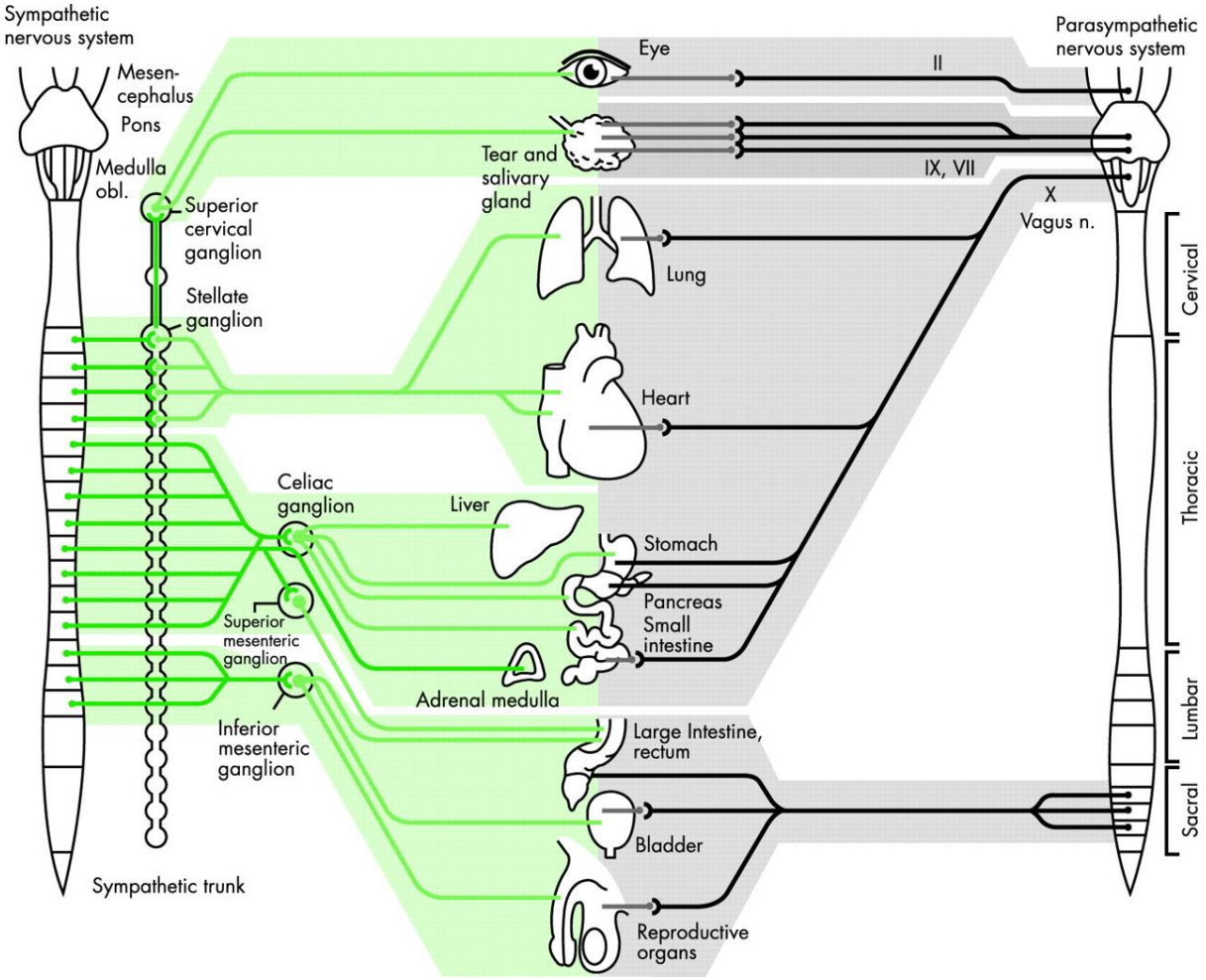


Figure 1.2. Figure outlining thoraco-lumbar sympathetic and cranio-sacral parasympathetic innervation of target organs. Reproduced from Janig W. In: Schmidt RF, Thews F, eds. Physiologie des menschen, 26th ed. Heidelberg: Springer-Verlag, 1995:340-69.

The cell bodies of sympathetic preganglionic neurons lie in the intermediolateral cell column of the thoracolumbar spinal cord from T1 to L3, and their axons synapse with postganglionic neurons at paravertebral or prevertebral sympathetic ganglia. In contrast, pre-ganglionic parasympathetic neurons lie within sacral spinal cord (S2-S4) and brainstem, with cell bodies of the parasympathetic system clustered within autonomic nuclei within the midbrain, pons and medulla. Postganglionic parasympathetic neurons are mainly located close to the target organs. Patients with autonomic failure can present with a wide spectrum of symptoms and signs, depending on the aetiology, including orthostatic hypotension, gastrointestinal, pupillary, secretomotor, sudomotor, bladder and sexual dysfunction (Table 1.1).

Table 1.1 Manifestations of autonomic failure

Systems affected	Symptoms and signs
Sympathetic adrenergic failure	Ptosis, meiosis
	Orthostatic hypotension
	Ejaculatory failure (in males)
Sympathetic cholinergic failure	Anhidrosis
Parasympathetic cholinergic failure	Dry eyes and mouth
	Dilated pupils with impaired reaction to light and accommodation
	Fixed heart rate
	Voiding difficulties/ urinary retention
	Constipation, bloating, atonic bowel
	Erectile failure (in males)

1.2 Classification of autonomic dysfunction

A historical approach to classifying autonomic dysfunction is into two major groups, 1) intermittent disorders where the autonomic nervous system is essentially normal except for malfunction at specific times, including autonomic mediated syncope and postural tachycardia syndrome, and 2) diseases where there is damage to the autonomic pathways, that is often, but not always, irreversible (Figure 1.3).[6] Amongst the diseases where there is damage to the autonomic pathways, these can be localised, such as Horner's syndrome, Holmes-Adie pupil, Frey's syndrome (gustatory sweating), or more generalised. Amongst the causes of generalised autonomic failure, there are several known diseases that are known to cause damage to the autonomic nervous system, and patients are typically labelled as having autonomic failure 'secondary' to these diseases. These include hereditary diseases, such as hereditary transthyretin amyloidosis, metabolic diseases such as diabetes and chronic renal failure, infections, like HIV and Chagas disease, and drugs and toxic causes, including chemotherapeutic agents and chronic alcohol excess. Other patients without a known pathology were previously grouped together with the label of 'primary' autonomic failure. These have been further subdivided based on their disease onset and progression into acute and chronic autonomic failure.

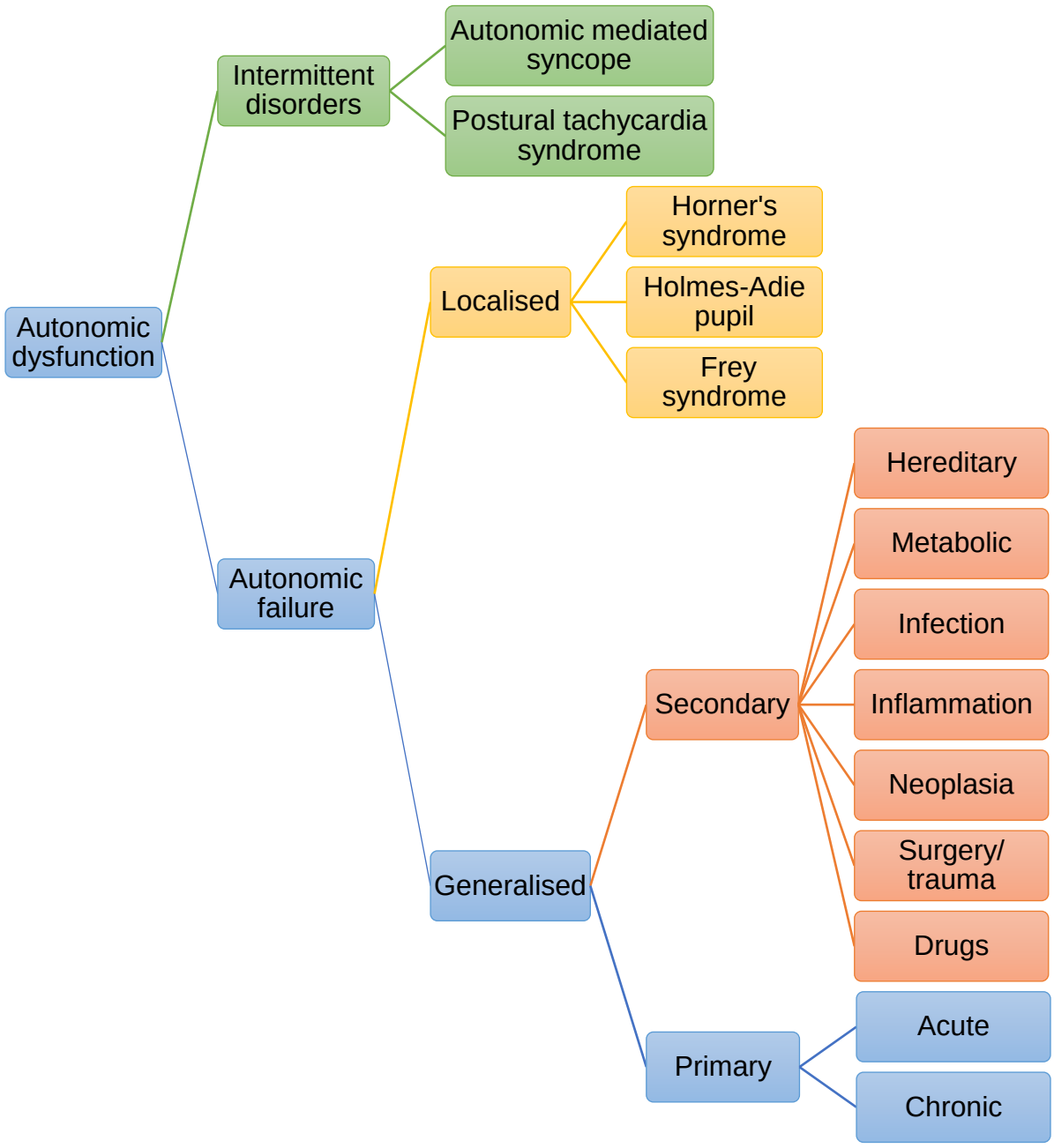


Figure 1.3. A historical approach to classifying autonomic diseases.

Under this historical classification, patients presenting with acute or subacute autonomic failure, which were thought to occur due to an inflammatory or auto-immune aetiology, were further subdivided according to clinical phenotype into pure pandysautonomia, pandysautonomia with neurological features, and pure cholinergic dysautonomia. The

primary chronic autonomic failure syndromes included patients where autonomic failure appeared to have resulted from selective neuronal degeneration, including a ‘pure’ form without any other neurological signs, ‘pure autonomic failure’ or PAF, and in the context of more widespread neurological symptoms, including multiple system atrophy (MSA) and the Lewy body diseases (LBD), Parkinson’s disease (PD) and Dementia with Lewy Bodies (DLB). This thesis will focus on the primary autonomic failure syndromes, including patients presenting with acute/subacute and chronic autonomic failure.

1.3 Clinical challenges in the diagnosis and treatment of patients presenting with autonomic failure

Patients presenting with autonomic failure at an early stage of their disease may represent a diagnostic challenge. The overall trajectory of their illness may not be evident, and they may not have developed the full spectrum of features to secure a definite diagnosis on clinical grounds alone, or indeed with the standard battery of investigations in routine practice.

Whilst a gradually progressive clinical presentation over several years is more suggestive of a neurodegenerative disease, and a post-infectious, subacute onset over months more in keeping with an autoimmune or inflammatory process, overlapping clinical features at initial presentation may lead to uncertainty between an autoimmune and neurodegenerative aetiology, with important implications for how these patients are treated.

A more comprehensive characterisation of the extent and degree of autonomic failure with multiple objective quantitative biomarkers may identify characteristic phenotypes to

help distinguish between different aetiologies at an earlier stage of the disease. Non-invasive, or minimally invasive biomarkers that can be repeated over time are also needed to quantify response to treatment.

1.4 Primary chronic autonomic failure syndromes: a historical perspective

Bradbury and Eggleston first described three patients with ‘idiopathic orthostatic hypotension’ in 1925, who presented with progressive syncopal attacks and anhidrosis, in the absence of other neurological symptoms and signs.[7] The patients had profound falls in blood pressure on orthostasis with a slow heart rate which was unaltered by physiological and pharmacological stimulation. The term ‘pure autonomic failure’ is now generally accepted for this syndrome, reflecting the fact that patients often have more widespread autonomic dysfunction, rather than isolated orthostatic hypotension. In 1960, Shy and Drager described two patients initially presenting with autonomic failure followed by motor involvement with central neurodegenerative changes on post-mortem in one of the patients.[8] They suggested that a primary neurodegenerative disease may be the underlying cause in some patients presenting with orthostatic hypotension, describing what became known as Shy-Drager syndrome, and now termed MSA. Patients with MSA develop severe autonomic failure with orthostatic hypotension, urinary retention or incontinence, and parkinsonism which is poorly responsive to levodopa, or a cerebellar syndrome. PD is the most common neurodegenerative movement disorder, and manifests with bradykinesia, cogwheel rigidity, resting tremor, postural instability. Some patients with otherwise typical PD develop prominent autonomic failure.

1.4.1 Pathophysiology of primary chronic autonomic failure syndromes

Pathologically, PD is characterised by the loss of dopaminergic neurons in the substantia nigra, with eosinophilic cytoplasmic neuronal inclusions, known as Lewy bodies. Lewy bodies have also been described in patients with dementia. Clinically, patients with dementia with Lewy bodies (DLB) present with a progressive dementia with fluctuating symptoms, extrapyramidal signs, prominent visual hallucinations, and increased sensitivity to neuroleptic medications. Studies in familial PD demonstrated mutations in genes for α-synuclein, and subsequent studies have demonstrated α-synuclein is a primary component of Lewy bodies and dystrophic Lewy neurites in sporadic PD and DLB. α-synuclein has also been shown to be a major component of the glial cytoplasmic inclusions that are found in MSA.[9]

Post-mortem studies of patients with PAF have demonstrated the presence of Lewy bodies predominantly in the autonomic ganglia and postganglionic neurons in the adrenal capsule, epicardial tissue and bladder wall,[10, 11] with relatively few Lewy bodies present in central structures such as the locus coeruleus and substantia nigra, and no significant central neuronal loss. PAF is now recognised a Lewy-body α-synucleinopathy, falling within a spectrum of neurodegenerative diseases including PD and DLB, with predominant peripheral autonomic nervous system involvement and minimal central nervous system involvement.[12] Post-mortem studies of patients with PAF, MSA and PD with AF showed severe loss of intermediolateral column cells of the sympathetic nervous system, suggesting this is a final common pathway in these neurodegenerative syndromes associated with autonomic failure.[13]

1.4.2 PAF phenoconversion or prodromal MSA?

In 1995, an international consensus committee defined PAF as an idiopathic sporadic disorder, characterised by OH, usually with evidence of more widespread autonomic failure, with no other neurological features.[14] The committee recognised that some patients presenting with PAF may later develop signs and symptoms of central nervous system evolvement, fulfilling criteria for other diseases such as MSA.

Subsequent natural history studies have shown that 12-34% of patients with PAF eventually develop progressive motor and/or cognitive symptoms and fulfil criteria for MSA, PD or DLB over 2-10 years.[15-17] Clinical features and biomarkers that have been associated with conversion from isolated autonomic failure to MSA include early age of onset in the 50s, preserved olfaction, severe bladder symptoms, need for catheterisation, supine noradrenaline >100pg/mL, supine heart rate >70bpm, orthostatic heart rate rise > 10bpm within 3 minutes, preserved cardiovagal function, preganglionic sudomotor dysfunction, normal cardiac ^{123}I-meta-iodobenzylguanidine scintigraphy (MIBG), and elevated CSF neurofilament light, and subtle motor signs not qualifying for parkinsonism or ataxia at initial assessment.[15-19]

The most recent 2022 Movement Disorders Society criteria for MSA outlined a new research category of ‘possible prodromal MSA’, which includes patients polysomnography-proven RBD (Rapid eye movement sleep Behaviour Disorder) or isolated autonomic failure, defined as urogenital failure with post-void residual volume >100ml or urinary urge incontinence, or neurogenic orthostatic hypotension within 10 minutes of standing or head-up-tilt, with subtle cerebellar or parkinsonian signs, not-requiring dopaminergic medication. The committee acknowledge this category has very

low specificity and will need further refinement with emerging data from prospective and biomarker studies.[20]

1.5 Acute and subacute autonomic failure: a historical perspective

In contrast to patients with primary chronic progressive autonomic failure, suggestive of a neurodegenerative aetiology, it was recognised that some patients presented with a more subacute onset of autonomic failure, suggestive of an autoimmune or inflammatory pathology. In 1994, Suarez et al described 27 patients with an acute or subacute onset of autonomic failure, using the label ‘idiopathic autonomic neuropathy’, drawing upon similarities with other acute idiopathic inflammatory neuropathies, including acute inflammatory demyelinating polyradiculopathy or Guillain-Barre syndrome. The patients frequently reported antecedent viral infections, typically demonstrating a monophasic disease course with gradual and often incomplete recovery, presumed to have a possible immune-mediated pathophysiology, with individual cases reported to benefit from empirical treatment with prednisone or intravenous immunoglobulin.[21] The clinical phenotype described was heterogenous. Cardiovascular and sudomotor autonomic testing revealed a spectrum of autonomic involvement ranging from isolated sudomotor impairment (11%), restricted cholinergic involvement (19%), predominant sympathetic deficits (22%), and varying degrees of pandysautonomia (48%). Some patients had distal sensory impairment (26%), mild limb weakness (22%), impaired deep tendon reflexes (33%), but most patients had normal nerve conduction studies (13/16, 81%). The wide spectrum of clinical phenotypes described in this early study suggests that patients with subacute, presumed autoimmune autonomic failure represent a heterogenous disease or set of diseases.

1.5.1 Discovery of the ganglionic acetylcholine receptor antibody

In 2000, Vernino et al reported the presence of antibodies binding to the nicotinic acetylcholine receptor on autonomic ganglia (gAChR) in 14/28 (50%) of patients with idiopathic autonomic neuropathy. Amongst the patients studied, prominent cholinergic failure, as indicated by impaired pupillary light responses in patients with severe parasympathetic dysfunction, most reliably predicted seropositivity for the gAChR antibody.[22] Subsequent studies demonstrated recapitulation of the disease phenotype in animal models using active and passive immunisation[23-25] and clinical recovery with immune treatment,[26-28] confirming the pathogenicity of the gAChR antibody and giving rise to the term ‘autoimmune autonomic ganglionopathy’ (AAG).

1.5.2 Clinical phenotype of gAChR-positive AAG

Subsequent studies explored the clinical phenotype and autonomic testing of patients with autonomic failure with and without the gAChR antibody to try establish the phenotype of patients with gAChR-positive AAG. Klein et al analysed ganglionic antibody levels in 121 patients with idiopathic neurogenic orthostatic hypotension, including patients with both subacute and chronic symptom onset.[29] They described 18 patients with positive gAChR antibody levels >50pM. Of the patients with very high antibody levels >1000pM had pandysautonomia with prominent cholinergic autonomic failure (including sicca complex, sudomotor impairment, abnormal pupillary light response, neurogenic bladder, constipation, and upper gastrointestinal symptoms, defined as early satiety, postprandial nausea, and vomiting) which was often subacute (71%) and with an antecedent presumed viral event (42%), although 28% had a more insidious onset with gradual progression. A subgroup (22%) with very low antibody levels (90 ± 10 pM) had

no significant cholinergic symptoms, insidious symptom onset, gradual progression and no antecedent events. The authors argued ganglionic antibody testing should be considered in a wider clinical spectrum of patients, including more chronic cases that would have been clinically indistinguishable from pure autonomic failure.

Sandroni et al subsequently compared the phenotype of patients with and without the gAChR antibody. gAChR-positivity was associated with a distinct clinical syndrome with subacute onset, sicca complex, abnormal pupillary light responses and lower gastrointestinal tract dysfunction.[30] The cardiovagal score of autonomic testing, based on heart rate response to deep breathing and Valsalva ratio, was the only objective clinical biomarker to differ significantly between the gAChR-positive and gAChR-negative groups, but of note, some autonomic domains, including secretomotor, pupillary and gastrointestinal function were not routinely tested. Tear and saliva production was not routinely measured in patients without sicca symptoms and gastrointestinal motility studies rarely performed overall.

Dedicated studies of pupillary function by Muppidi et al in patients with gAChR-positive AAG showed a unique finding of premature pupillary redilation within a standard 2-second light impulse.[31] This phenomenon, termed pupil fatigue, was postulated to be a clinical correlate of defective synaptic transmission at the autonomic ganglia, and was not seen in healthy controls or 110 consecutive patients with other autonomic diseases.

In 2018, Cutsforth-Gregory at al reported a retrospective review of 289 patients seen at the Mayo Clinic between 1997-2015 with gAChR antibody levels ≥50pM with contemporaneous autonomic testing. They found that elevated gAChR antibody levels ≥400pM were moderately sensitive and highly specific for severe autonomic failure, but

low levels <200pM did not predict the presence or absence of autonomic failure, suggesting that low levels <200pM in the absence of autonomic failure were usually of little clinical signifcance.[32]

1.5.3 Seronegative autoimmune autonomic failure

While there appears to be a reproducible clinical phenotype for patients with high titres of the gAChR antibody, studies of patients with autonomic failure due to a presumed autoimmune aetiology without the gAChR antibody have been more heterogenous, partly due to difficulties with and differences in defining seronegative autoimmune autonomic failure.

In 2009, Iodice et al described the clinical phenotype and response to immunotherapy for six patients with a clinical diagnosis of autoimmune autonomic failure, four with the gAChR antibody and two without. In the absence of the ganglionic antibody, patients with idiopathic dysautonomia were required to have orthostatic hypotension, significant gastrointestinal symptoms, and severe autonomic failure on standardised cardiovascular and sudomotor autonomic testing to be considered seronegative putative autoimmune autonomic failure. Additional suggestive criteria included pupillary involvement, prior antecedent event, evidence of tissue inflammation and subacute onset.[27] The two seronegative patients, by definition, had severe adrenergic, cardiovagal and sudomotor autonomic failure, but neither had abnormal pupils or significant cholinergic symptoms (present in 3 of the 4 gAChR-positive patients). Both patients had a length-dependent axonal neuropathy on NCS/EMG (normal in all the seropositive patients), and one patient, who presented with numbness in the feet, face and tongue, also had evidence for a right trigeminal neuropathy. Both seronegative patients had marked objective

improvements on repeated autonomic testing after intravenous immunoglobulin (IVIg), with clear improvement in adrenergic and cardiovagal function. One also had an improvement postganglionic sudomotor function as assessed by quantitative sudomotor axon reflex testing. The authors argued that, as with other autoimmune neurological diseases like myasthenia gravis, gAChR-negative patients with the appropriate phenotype suggestive of autoimmune autonomic failure should treated with immunotherapy.

Golden et al described six patients with subacute autonomic failure and negative gAChR antibodies.[33] Three patients had severe neuropathic pain with reduced pinprick sensation and two also had impaired vibration/joint position sense. One patient had absent deep tendon reflexes, a length-dependent axonal sensorimotor polyneuropathy on NCS/EMG, elevated CSF protein, and axonal loss and scattered inflammatory infiltrates on sural nerve biopsy. Cardiovascular testing revealed severe sympathetic autonomic failure in all patients, with relative sparing of parasympathetic cardiovagal function in 5 patients. Pupillometry revealed a mild parasympathetic deficit in one patient and mixed sympathetic and parasympathetic deficits in two patients. None of the patients had premature pupillary redilation characteristic of gAChR-positive AAG. Quantitative sudomotor axon reflex testing (QSART) showed impaired postganglionic sudomotor function in all 6 patients.

Two patients reported no effect with plasma exchange or rituximab, but improved orthostatic and urinary symptoms with steroids and azathioprine, with relapse upon discontinuation of treatment. One patient had non-sustained improvements in orthostatic hypotension with pulsed intravenous methylprednisolone but improved with

mycophenolate and hydroxychloroquine, remaining stable after stopping mycophenolate after 1.5 years. Three patients had no clear benefit with various treatments including plasma exchange, IVIg, rituximab, steroids and steroid sparing agents: two had a gradual recovery after 1-1.5 years, and the third patient was lost to follow up. The authors argue these patients have a distinct phenotype from gAChR-positive AAG, with predominant sympathetic autonomic failure. They argued the improvement with steroids in some patients above expected from simply increased intravascular volume (for example improved urinary function) and lack of response to antibody targeted therapies suggested a possible cell-mediated or inflammatory autoimmune aetiology, rather than an antibody-mediated disease. An important limitation of this retrospective study is the lack of objective documentation of recovery using quantitative autonomic testing before and after immunotherapy. It is not clear whether the reported improvements in symptoms in some of the patients were clearly temporally linked to immune therapy or plateauing of a monophasic illness.

1.6 Skin biopsies in peripheral and autonomic neuropathies

Skin biopsies are a minimally invasive method of studying peripheral sensory and autonomic nerves. Indirect immunofluorescence techniques with pan-neuronal marker protein G product 9.5 (PGP) can be used visualise nerve bundles arising from the subepidermal neural plexuses penetrating the basement membrane and innervating the epidermis in a vertical orientation as naked axons. Intraepidermal nerve fibres (IENF) are the terminal endings of small C and A-delta fibres originating from the dorsal root ganglia and convey pain and thermal stimuli. They are typically found at a higher density at proximal sites compared to distal sites. Small fibre neuropathies secondary to toxic and

metabolic aetiologies typically demonstrate a length-dependent pattern, whereas a non-length dependent or ganglionic symptoms may suggest an autoimmune process.[34] Repeat skin biopsies can be used to study the progression or recovery of disease over time, with previous studies demonstrating improvements in IENF density in patients with impaired glucose tolerance following lifestyle changes, in patients with hypothyroidism following hormone replacement therapy, and in hereditary sensory and autonomic neuropathy after l-serine treatment.[35-37]

Skin biopsies also allow the study of peripheral autonomic nerves. In healthy individuals, cutaneous sweat glands can be easily identified as a dense network of PGP-ir fibres encircling sweat tubules. Most sudomotor fibres are reactive to vasoactive intestinal peptide (VIP), a cholinergic marker. In hairy skin, arrector pili muscles connect hair follicles to the basement membrane. They have a vestigial thermoregulatory role in humans. Pilomotor nerve fibres run in parallel to the smooth muscle fibres, and are mainly adrenergic, reactive to dopamine beta hydroxylase (DβH), with some cholinergic VIP-ir fibres also present. Arteriovenous anastomoses are complex structures which direct cutaneous blood flow towards or away from the capillary circulation, contributing to heat dispersion and thermoregulation. They are typically densely innervated by adrenergic, DβH-ir fibres, with less dense networks of cholinergic, VIP-ir fibres. Various semi-quantitative and quantitative methods have been used to assess sudomotor, pilomotor and vasomotor innervation.[38-40] Previous studies have shown patients with autonomic neuropathies due to various aetiologies had reduced autonomic innervation compared to healthy controls[39] and patients with idiopathic small fibre neuropathies without autonomic involvement.[41]

1.6.1 Skin biopsies in PAF and other synucleinopathies

Skin biopsies have also been used to study patients PAF and other α-synucleinopathies. Donadio et al found patients with PAF had reduced adrenergic and cholinergic fibres compared to patients with MSA and controls, with denervation correlating with disease duration.[42] In contrast, patients with MSA had relatively preserved autonomic innervation, in keeping with a predominantly central autonomic pathology. Of note, the patients with PAF had a significantly longer disease duration than the patients with MSA, but the authors performed a subgroup analysis comparing 6 patients from each group with similar disease duration and found the autonomic innervation was still higher in the MSA group.

1.6.2 Cutaneous α-synuclein as a potential biomarker for PAF and other α-synucleinopathies

Cutaneous α-synuclein has been studied as a potential diagnostic biomarker for PAF and other α-synucleinopathies. Donadio et al initially showed that misfolded phosphorylated α-synuclein (p-syn) deposits were present in all patients with PAF (n=9) but not in patients with acquired autonomic neuropathies (n=12) or healthy controls (n=15).[43] Somatic and autonomic innervation was reduced in both patients with PAF and acquired autonomic neuropathies compared to controls. They later compared p-syn distribution in PAF and idiopathic PD, finding p-syn deposits in all samples from patients with PAF but only 49% of samples from patients with PD, in keeping with greater peripheral nervous system involvement in PAF. Patients with PD had greater p-syn positivity at proximal compared to distal sites.[44] A further study comparing patients with PD with and without neurogenic orthostatic hypotension (OH) showed p-syn was more likely to be present in

samples those with OH compared to those without, again with greater p-syn positivity at proximal sites.[45]

A subsequent study comparing patients with MSA-Parkinsonian subtype (MSA-P) and PD with OH found p-syn in all PD with OH patients but only 72% of MSA-P patients, mainly at distal sites. In MSA-P, p-syn deposits were primarily found in the somatic nerves of the subepidermal plexuses, whereas in PD with OH, p-syn was widely distributed in the autonomic fibres.[46] Autonomic innervation was relatively preserved in MSA-P compared to PD with OH. Of note, patients with PD with OH were significantly older, with a longer disease duration. In another study p-syn was found in 75% of skin samples from patients with DLB, with patients with autonomic symptoms showing greater p-syn positivity (97% vs 71%) and reduced autonomic innervation compared to those without autonomic symptoms.[47] A recent study in patients with early PD and MSA-P, within 2 years of motor onset, showed higher p-syn deposits in patients with PD compared to MSA-P.[48]

In MSA, α-synuclein typically accumulates in oligodendroglial cells as glial cytoplasmic inclusions, rather than in neurons as Lewy bodies and Lewy neurites in PD and the other synucleinopathies. Recently, Donadio et al also found p-syn deposits in Remak non-myelinated Schwann cells in all MSA patients positive for p-syn except for two patients with very scant p-syn positivity, and none of the patients with PD/DLB and healthy controls, giving 74% sensitivity and 100% specificity for detecting MSA.[49]

1.7 Differential diagnosis in a patient presenting with autonomic failure

At initial assessment of a patient presenting with autonomic failure, there may be a clear history and additional clinical signs consistent with a neurological or systemic disease

associated with autonomic failure, such as longstanding diabetes with suboptimal glycaemic control and evidence of peripheral neuropathy, retinopathy, nephropathy, and other end organ damage. There may be evidence of an emerging peripheral neuropathy, bilateral carpal syndrome, spinal stenosis, cardiac, ophthalmic, or other systemic features and a family history consistent with inherited amyloidosis or specific features of another rare genetic disease. Patients may describe previous infection, trauma, surgery, radiotherapy, chemotherapy, or exposure to other toxins prior to the development of symptoms. Clinicians may uncover, on direct enquiry, a history of RBD or hyposmia, or detect subtle motor signs on examination that may be difficult to distinguish from normal aging at initial assessment but suggest a more widespread neurodegenerative disease may be emerging (Figure 1.4).[15, 17, 18, 50]

A systematic interrogation of the onset, severity and nature of the patient's autonomic symptoms may evoke a particular diagnosis. A subacute post-infectious explosive onset of pandysautonomia with severe sicca syndrome, upper gastrointestinal symptoms or pseudo-obstruction and tonic pupils is highly suggestive of an autoimmune aetiology,[22] rapidly progressive autonomic failure with early genitourinary symptoms and requirement for catheterisation may point towards evolving MSA, [16-18, 51] whereas insidiously progressive orthostatic intolerance over several years with hypohidrosis is suggestive of the relatively benign, peripherally-predominant alpha-synucleinopathy, PAF.[51]

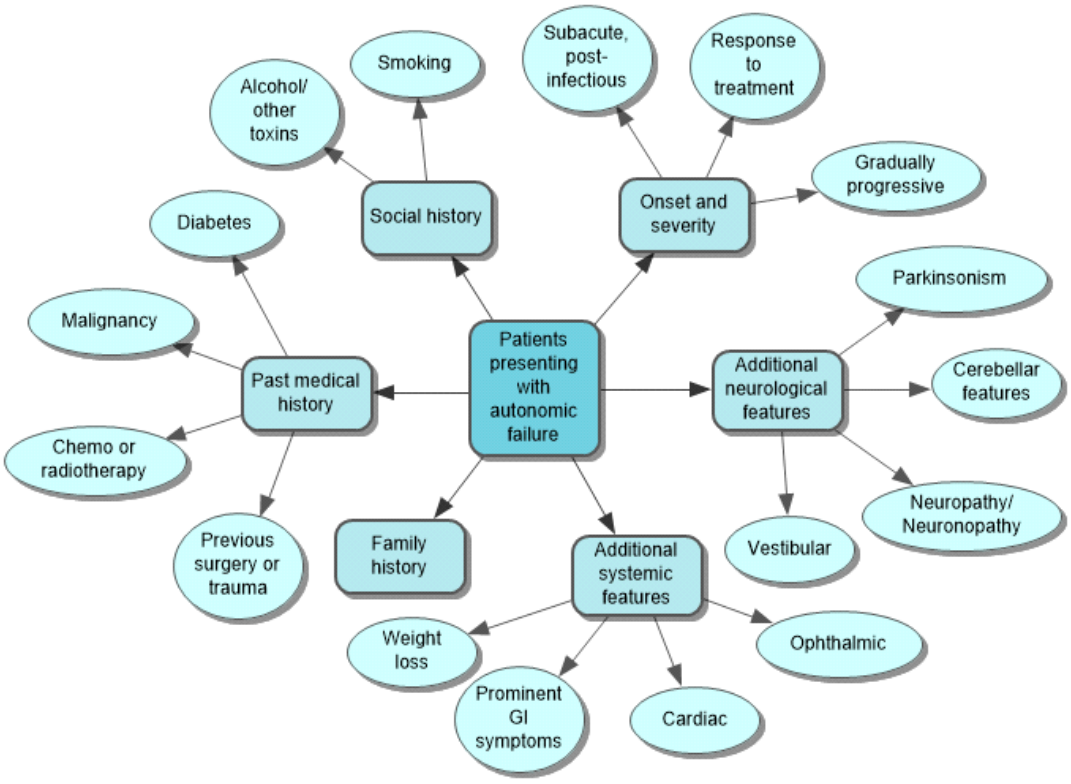


Figure 1.4. Clinical clues in a patient presenting with autonomic failure. A careful clinical assessment may reveal a history of previous potential triggers or insults to the autonomic nervous system, a relevant family history, extrapyramidal signs, evidence of a peripheral neuropathy, or other systemic features.

However, even to experienced clinicians, patients presenting with autonomic failure at an early stage of their disease represent a diagnostic challenge, as the overall trajectory of their illness may not be evident, and they may not have developed the full spectrum of features to secure a definite diagnosis on clinical grounds alone, or indeed with the standard battery of investigations in routine practice. A more comprehensive characterisation of the extent and degree of autonomic dysfunction with multiple objective quantitative biomarkers may provide clues regarding the localisation and aetiology of the underlying disease at an earlier stage of the illness.

1.8 Summary of current gaps in knowledge

Standard cardiovascular and sudomotor autonomic testing performed in most autonomic laboratories are not able to capture the full spectrum of autonomic deficits, particularly the cholinergic deficits that are prominent in patients with the gAChR antibody including pupillary function, tear and saliva production, bladder and bowel function. A panel of biomarkers assessing multiple autonomic domains may help to identify distinctive clinical phenotypes in patients where the underlying aetiology is unclear, enabling earlier diagnosis and delivery of targeted disease modifying treatment, where available. In addition, objective quantitative autonomic assessments before and after response to immune treatment in patients with autoimmune autonomic ganglionopathy to allow clinicians to plan individualised treatment.

1.9 Study hypotheses

1. Patients with gAChR-positive AAG will demonstrate a distinct phenotype, characterised by subacute widespread sympathetic and parasympathetic autonomic failure.
2. Patients with gAChR-negative autoimmune autonomic failure represent a heterogenous set of diseases, some of whom may demonstrate selective impairments of the autonomic nervous system, with either sympathetic adrenergic predominant or parasympathetic cholinergic predominant autonomic failure, and evidence for a peripheral large fibre neuropathy or sensory neuronopathy.
3. Patterns of autonomic denervation will differ between gAChR-positive and gAChR-negative autoimmune autonomic failure, in keeping with differences in their clinical phenotype.

4. Amongst patients with α-synucleinopathies, patients with PAF will demonstrate greater postganglionic dysfunction and denervation, in keeping with a peripherally predominant α-synucleinopathy, in contrast to patients with MSA, a centrally predominant α-synucleinopathy.
5. Analysis of p-syn deposits in cutaneous peripheral nerves can help to distinguish between patients with peripherally and centrally predominant α-synucleinopathies, and non-synucleinopathy related diseases.
6. Cutaneous autonomic denervation will correlate with quantitative markers of autonomic failure and patient reported outcomes.

1.10 Aim and objectives

Aim:

The overarching aim of this thesis was to explore and define key biomarkers or clusters of biomarkers to improve the understanding, diagnosis and treatment of autonomic failure due to autoimmune and neurodegenerative aetiologies. The following objectives outline in detail how this aim will be achieved:

Objectives:

1. Retrospectively evaluate the autonomic testing profile at first presentation in patients with autoimmune and neurodegenerative autonomic failure at a national autonomic referral centre over twenty years.
2. Prospectively recruit and systematically study patients presenting with autonomic failure with a multimodal autonomic testing protocol, punch skin biopsies, and

standardised questionnaires to capture autonomic symptom burden and impact on quality of life.

3. Define clusters of clinical, autonomic, blood and cutaneous biomarkers to aid the early diagnosis in patients presenting with autonomic failure, and to quantify recovery after immune treatment in patients with autoimmune autonomic failure.
4. Identify clinical features and biomarkers that distinguish between different α-synucleinopathies.
5. Identify clinical features and biomarkers that predict phenoconversion in patients presenting with pure autonomic failure.
6. Study the relationship between cutaneous p-syn deposition, peripheral autonomic denervation, quantitative markers of autonomic failure, and patient reported outcomes.

1.11 Funding

1. I received salary support from The Guarantors of Brain Entry Fellowship.
2. The purchase of the pupillometer for our prospective studies was kindly supported by a grant from the National Brain Appeal Small Acorns Fund.
3. I received a travel grant from the Department of Brain, Repair and Rehabilitation which enabled me to undergo training with and process my samples at Professor Maria Nolano's Skin Biopsy laboratory in Italy.
4. The studies on patients with α-synucleinopathies were partly financed by a grant from the Italian Ministry of Health "Ricerca Finalizzata 2013," project code PE-2013-02359028.

1.12 Ethical approval

Ethical approval for this study was granted by the London Bridge Research Ethics Committee, REC reference 16/LO/1656). All prospective study participants provided written informed consent to participate in this study. The study was conducted in accordance with Good Clinical Practice guidelines and the World Medical Association Declaration of Helsinki.

Chapter 2. General methods

2.1 Patient recruitment

Patients were recruited for the prospective studies in this thesis from patients referred to the Autonomic Unit at the National Hospital for Neurology and Neurosurgery between 2018 and 2021. For retrospective studies, patient records and clinical testing of patients referred to the Autonomic Unit between 1987 and 2021 were reviewed to identify patients meeting inclusion criteria for individual studies.

2.2 Patient groups

2.2.1 gAChR-positive AAG

Patients with subacte autonomic failure with elevated gAChR antibody levels >100pM were diagnosed with gAChR-positive AAG.

2.2.2 gAChR-negative autoimmune autonomic failure

Patients presenting with a subacute onset of autonomic failure, with a monophasic course with plateau phase, or with recovery either spontaneously or following immune therapy, with negative gAChR antibodies, were diagnosed with gAChR-negative autoimmune autonomic failure.

2.2.3 PAF

Patients with progressive autonomic failure with prominent orthostatic hypotension, usually with evidence of more widespread autonomic failure, with no other neurological features, other than RBD (REM sleep behaviour disorder), typically with reduced plasma noradrenaline levels, were diagnosed with pure autonomic failure, in accordance with 1996 international consensus criteria.[14]

2.2.4 MSA

Patients with at least clinically probable MSA in accordance with international consensus criteria were studied.[20, 52] The most recent 2022 Movement Disorder Society consensus criteria defines this as patients with symptom onset >30 years, a negative family history, and a progressive disease course with at least two core clinical features, summarised in Table 2.1.

Table 2.1. Core clinical features for clinically probable MSA

Core clinical features	Clinically probable MSA
Autonomic dysfunction	At least one of: • Unexplained voiding difficulties with post-void urinary residual volume • Unexplained urinary urge incontinence • Neurogenic OH with ≥20/10mmHg blood pressure drop within 10 minutes of standing or head-up tilt
Parkinsonism	• Bradykinesia, slowness of movement and decrement in amplitude or speed or progressive hesitations/ halts as movements are continued plus • Rigidity, velocity-independent resistance to passive movement or • Tremor, rhythmic or arrhythmic involuntary movement in arms or legs
Cerebellar syndrome	At least one of gait ataxia, limb ataxia, cerebellar dysarthria, or oculomotor dysfunction (sustained gaze-evoked horizontal or downbeat nystagmus or saccadic hypermetria)

2.2.5 PD

Patients with at least clinically probable PD in accordance with 2015 international consensus criteria as outlined by the Movement Disorders Society were included.[53] In summary, these are defined as patients with parkinsonism, with any red flags counterbalanced by supportive criteria as outlined in Table 2.2

Table 2.2. Supportive criteria and red flags for a diagnosis of PD.

Supportive criteria for PD	Red flags
Clear and dramatic beneficial response to dopaminergic therapy	Complete absence of progression of motor signs and symptoms over ≥5 years
Levodopa induced dyskinesia	Rapid progression of gait impairment with regular wheelchair use within 5 years
Rest tremor of a limb	Early bulbar dysfunction within 5 years
Olfactory loss or cardiac sympathetic denervation on MIBG scintigraphy	Inspiratory respiratory dysfunction
	Severe autonomic failure within 5 years • OH ≥ 30/15mmHg within 3 minutes standing • Severe urinary incontinence or retention
	Recurrent falls due to impaired balance within 3 years
	Disproportionate anterocollis/contractures within 10 years
	Absence of common non-motor features despite 5 years duration, including sleep, autonomic, psychiatric dysfunction or hyposmia
	Unexplained pyramidal signs
	Bilateral symmetric parkinsonism throughout disease course

2.2.6 DLB

Patients with at least probable DLB according to the 2017 DLB Consortium consensus report were included.[54] These were defined as patients with progressive cognitive decline of sufficient magnitude to interfere with usual daily activities, with two or more core clinical features; or one core clinical feature with at least one indicative biomarker as summarised in Table 2.3.

Table 2.3. Core clinical features and indicative biomarkers for DLB.

Core clinical features for DLB	Indicative biomarkers
Fluctuating cognition	Reduced dopamine transporter uptake in basal ganglia on SPECT (single-photon emission computerized tomography) or PET (Positron emission tomography)
Recurrent visual hallucinations	Abnormal MIBG scintigraphy
RBD	Polysomnographic confirmation of REM sleep without atonia
≥1 of bradykinesia, rest tremor, or rigidity	

2.2.7 Exclusion criteria

I excluded patients with autonomic failure secondary to systemic diseases including diabetes mellitus, chronic renal failure, amyloidosis, and other rare genetic diseases, or exposure to chemotherapy, radiotherapy, or sympathectomies.

2.3 Patient testing

2.3.1 Study preparation

Autonomic testing was performed in controlled ambient temperatures at autonomic laboratories in the National Hospital for Neurology and Neurosurgery. Medications affecting autonomic testing were stopped at least 5 half-lives prior to testing.

Before testing, patients were asked to refrain from

1. Heavy exercise for 24 hours
2. Alcohol ingestion for 12 hours
3. Caffeine intake for 12 hours
4. Eating for 4 hours

2.3.2 Cardiovascular autonomic testing

Beat-to-beat measurements of blood pressure and heart rate were recorded and analysed with Labchart 8 Pro software (AD Instruments).[55]

2.3.2.1 Stand

Patients were rested in the supine position for 10 minutes. They were then asked to stand with measurements of blood pressure and heart rate taken after 1, 3, and 5 minutes. Some patients were not able to tolerate a full 5 minutes standing due to severe orthostatic hypotension. The fall in systolic blood pressure in mmHg was divided by the maximum time tolerated standing (up to 5 minutes) to give the orthostatic intolerance ratio on stand (OIR-stand).

2.3.2.2 Head-up tilt

Patients were rested in the supine position for 10 minutes on a horizontal tilt table. The tilt table was then moved into a 60° head-up position for up to a maximum of 10 minutes. Tilt was terminated early if patients developed severe orthostatic hypotension with symptoms and signs suggesting imminent syncope (Figure 2.1). The fall in systolic blood pressure in mmHg was divided by the maximum time tolerated on head up tilt (up to 10 minutes) to give the orthostatic intolerance ratio on tilt (OIR-tilt).

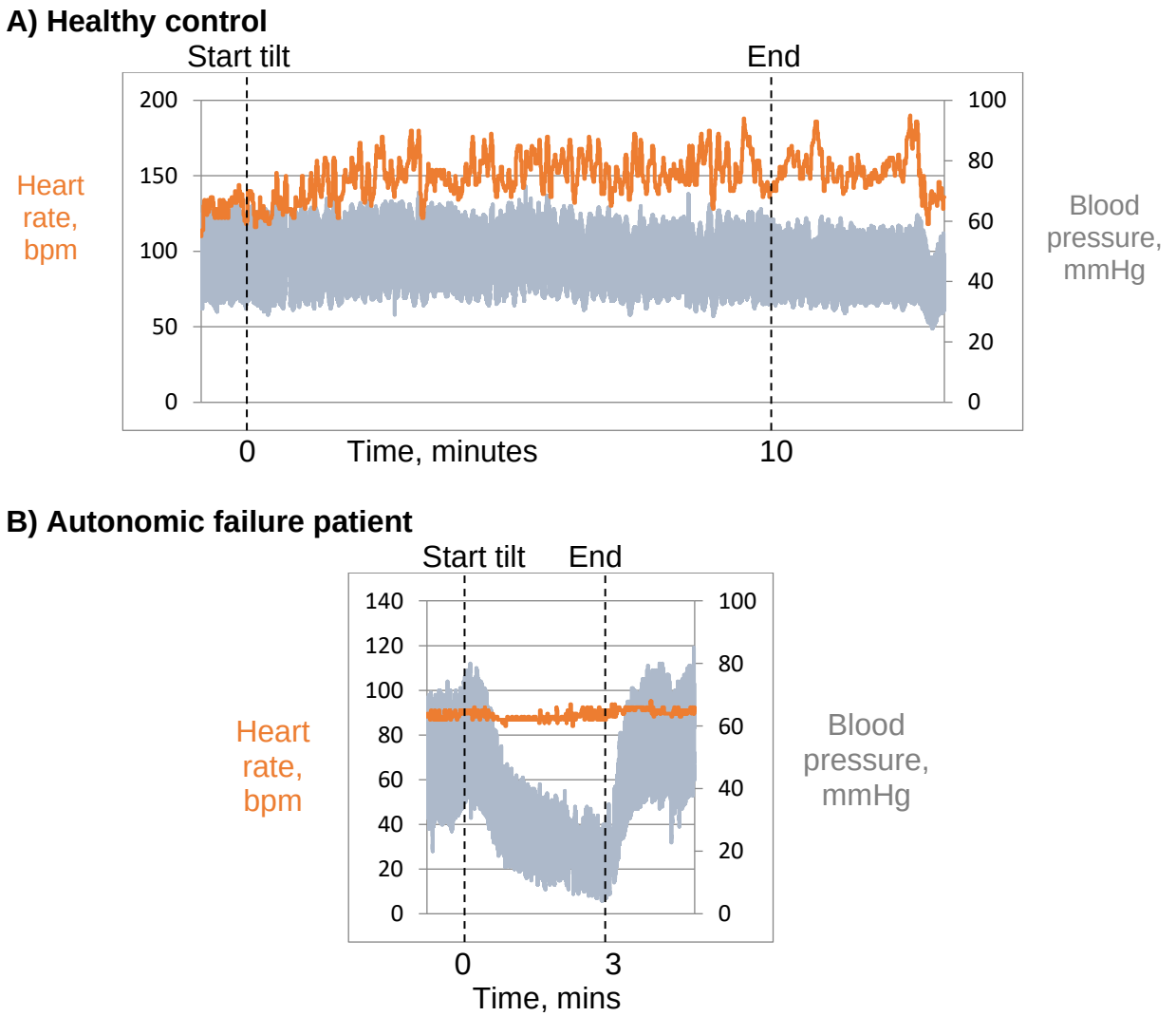


Figure 2.1. Beat-to-beat blood pressure and heart rate recordings on head-up tilt in a healthy control (A) and a patient with autonomic failure (B), demonstrating severe orthostatic hypotension requiring early termination of tilt at 3 minutes, with no compensatory heart rate increase (B).

2.3.2.3 Handgrip isometric exercise testing

Patients were asked to perform maximum voluntary handgrip on a manual sphygmomanometer pump on 3 occasions to establish individual maximum contraction values. They were then rested and asked to perform a sustained handgrip at 33% of their maximum contraction for 3 minutes (Figure 2.2).

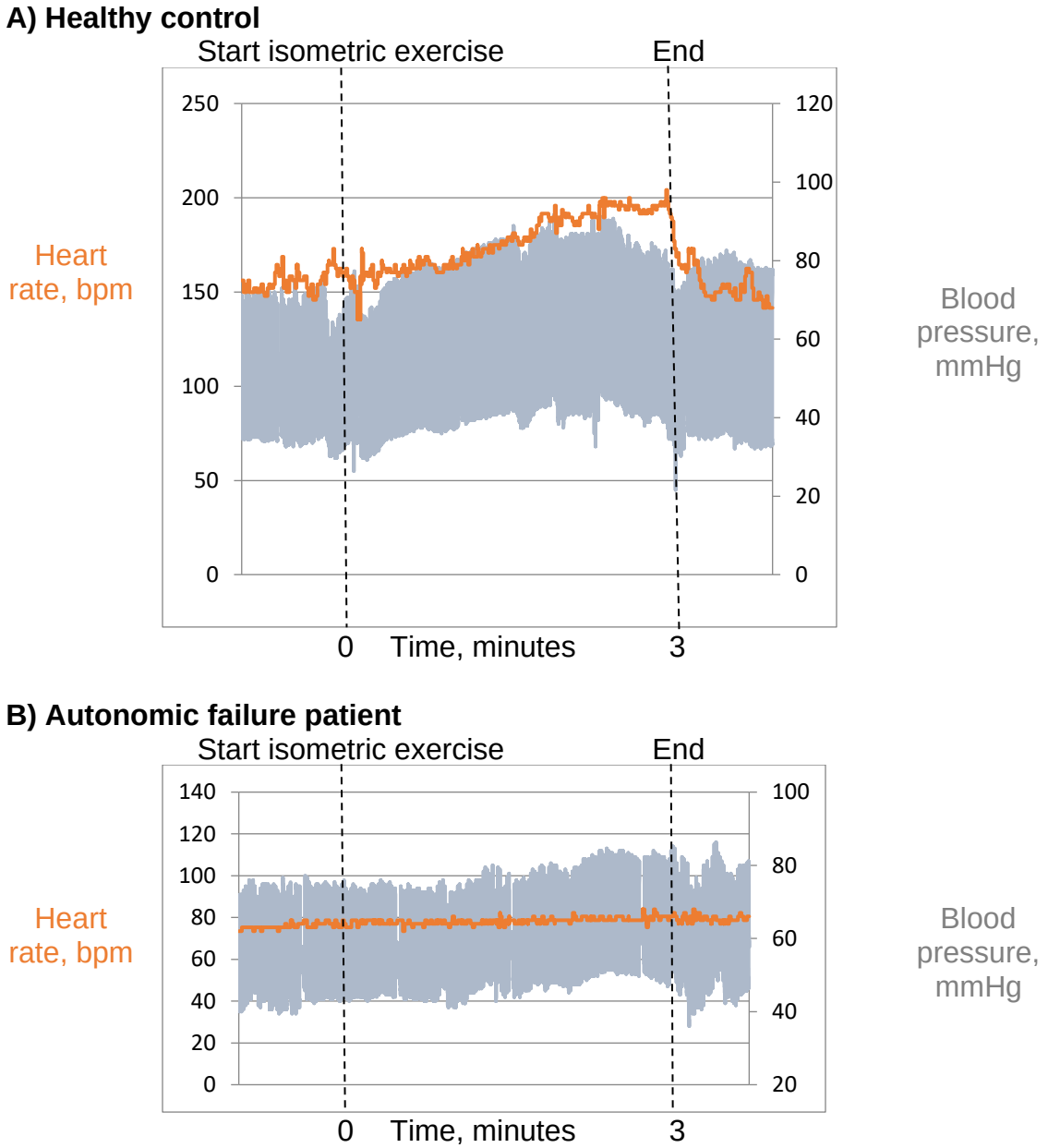


Figure 2.2. Beat-to-beat blood pressure and heart rate recordings before and after isometric exercise in a healthy control (A) and a patient with autonomic failure (B), demonstrating a lack of rise in blood pressure and heart rate.

2.3.2.4 Heart rate response to deep breathing (HR_{DB})

Patients were asked to breathe deeply at a rate of 6 breaths/minute for 1 minute. The difference in heart rate between inspiration and expiration was noted, and the average for 6 breaths calculated (Figure 2.3) to give the heart rate response to deep breathing (HR_{DB}), an index of cardiovagal function.

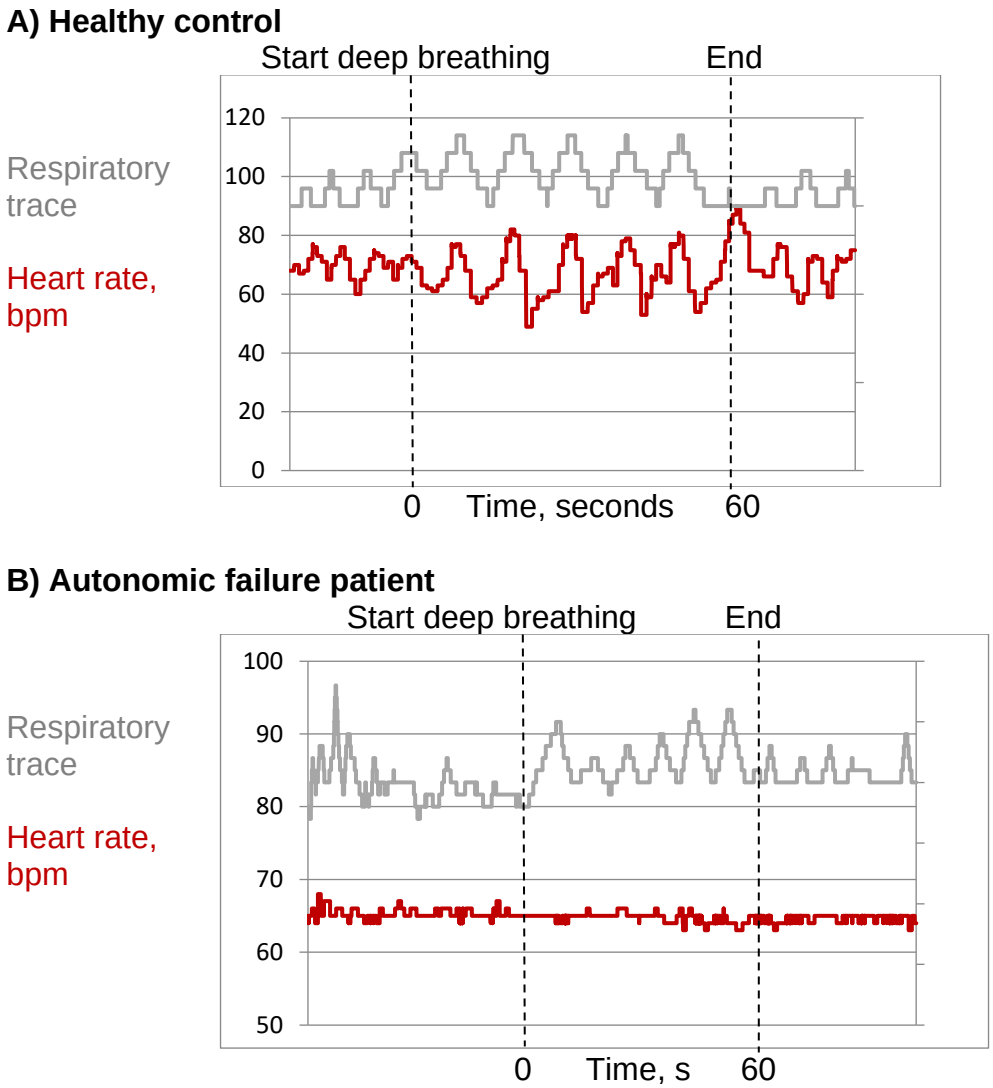


Figure 2.3. Beat-to-beat heart rate recordings during deep breathing in a healthy control (A) and a patient with autonomic failure (B), demonstrating a lack of heart rate variability with deep breathing.

2.3.2.5 Valsalva manoeuvre

Patients performed a forced expiration against a fixed expiratory pressure of 40mmHg for 10-15 seconds. For subjects with a ‘flat top’ response, who did not develop an early phase II fall in blood pressure, this was repeated with the subjected tilted to 20°, or 40°, if necessary, until a significant fall in blood pressure was obtained. The manoeuvres were repeated until two reproducible beat-to-beat recordings were obtained.

The four main phases of the Valsalva manoeuvre are shown in Figure 2.4. In Phase I, blood pressure rises transiently due to increased intrathoracic pressure causing mechanical compression of the aorta. In early phase II (phase II_E), reduced venous return and stroke volume lead to a fall in cardiac output. In a normally functioning autonomic system (A), increased sympathetic discharge causes arteriolar vasoconstriction and increased total peripheral resistance, arresting the fall in blood pressure, phase II late (II_L). Phase III occurs when the subjects stop forced expiration, and the fall in intrathoracic pressure leads to a fall in blood pressure. This is mechanical, like phase I. Finally, in phase IV, when venous return and cardiac output return to normal, the blood pressure transiently overshoots above baseline values due to ongoing arteriolar vasoconstriction. In a patient with adrenergic failure (B), there is a loss of phase II late blood pressure recovery, phase IV blood pressure overshoot, and a delay in blood pressure recovery to the baseline after the Valsalva manoeuvre.

The Valsalva ratio (VR) was calculated by dividing maximum heart rate developing during Phase II of the Valsalva manoeuvre over the minimum heart rate occurring within 30 seconds of the peak heart rate. The blood pressure recovery time (PRT) was defined

as the time taken for the systolic blood pressure to recover from Phase III back to the baseline, an index of sympathetic adrenergic function.[56, 57]

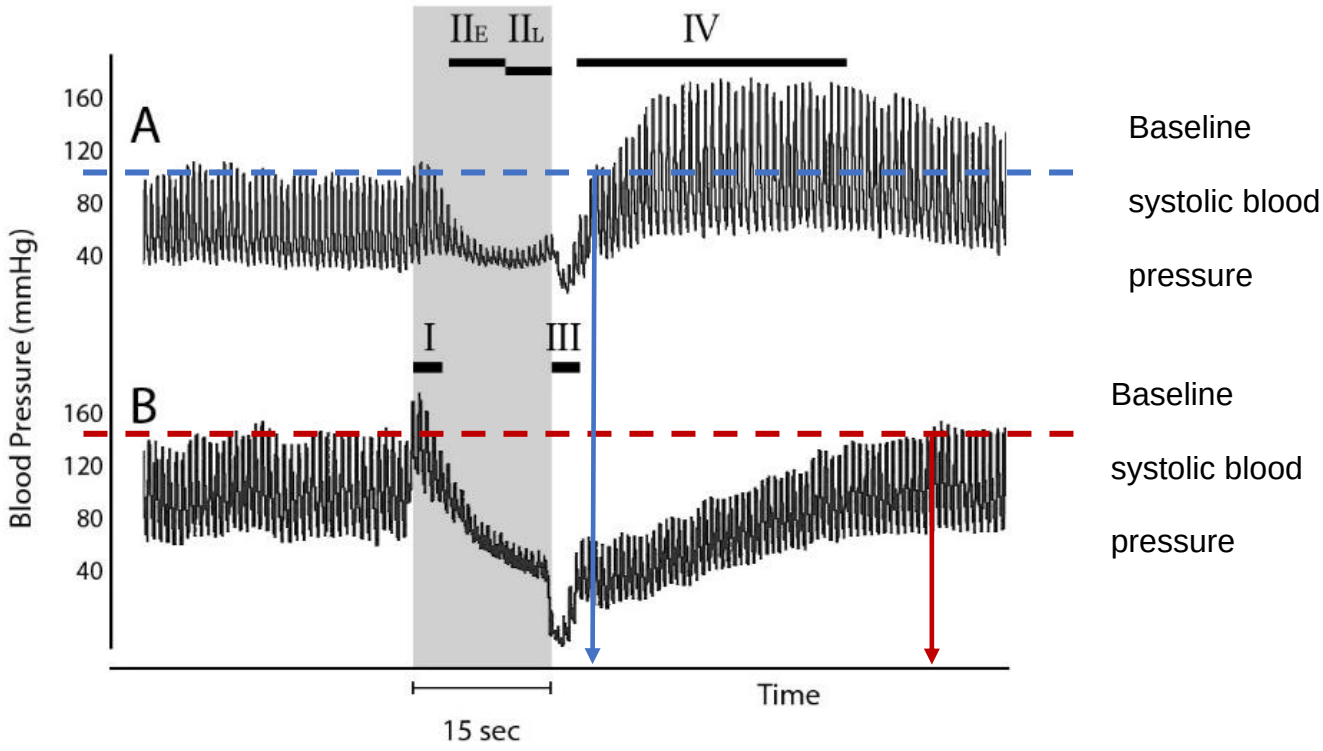


Figure 2.4. Beat-to-beat blood pressure in a healthy control (A) and patient with adrenergic autonomic failure (B). In B, there is an absence of phase II late blood pressure recovery, phase IV blood pressure overshoot, and a delay in the time taken for systolic blood pressure to return to baseline values after the Valsalva manoeuvre. Adapted from Cheshire et al, 2021, Clinical Neurophysiology.[56]

2.3.2.6 Plasma noradrenaline testing

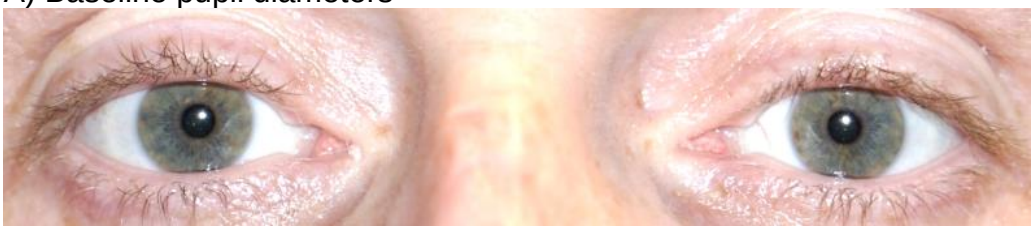
Blood was collected via intravenous forearm catheter in the supine and tilted position to assess for plasma catecholamines, including noradrenaline, with high-performance liquid chromatography. Plasma noradrenaline (norepinephrine) gives a measure of sympathetic neural activity. Patients with neurogenic orthostatic hypotension have attenuated orthostatic increments in plasma noradrenaline.[58] Supine plasma noradrenaline is typically reduced in PAF, in keeping with postganglionic noradrenergic denervation, and relatively preserved in MSA.[14, 51, 59]

2.3.3 Pupillometry

Baseline pupillary dark diameters and responses to stimulation with white light and topical pharmacological agents were recorded with a custom built infrared pupillometer[60] or commercially available devices (Procyon P3000, Konan RAPDx, or Neuroptics DP2000). Impaired pupillary constriction to light and accommodative effort, and cholinergic supersensitivity (pupillary constriction with dilute 0.125% pilocarpine) indicated parasympathetic dysfunction.

Delayed pupillary redilation following a light impulse to ¾ of baseline diameter, a lack of response to 4% cocaine, or adrenergic supersensitivity (abnormal dilation with 0.5% apraclonidine) indicated sympathetic dysfunction Figure 2.5.[61]

A) Baseline pupil diameters



B) After 0.5% apraclonidine

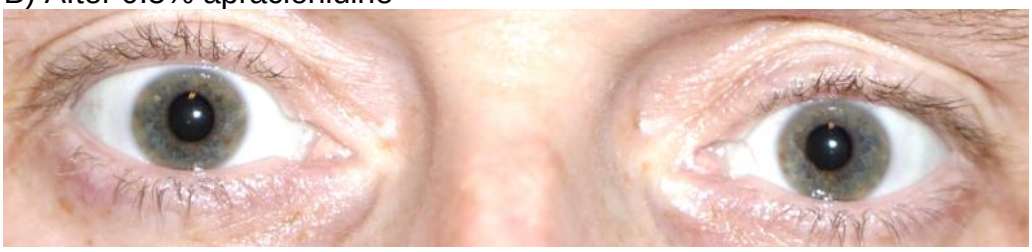


Figure 2.5. Abnormal pupillary dilation in both pupils after 0.5% apraclonidine drops indicating adrenergic denervation supersensitivity.

From May 2019, patients were also examined with a 2-second light stimulus to assess for premature pupillary redilation within the light stimulus, termed pupil fatigue. This is a unique phenomenon previously reported only in patients with gAChR-positive AAG.[31]

2.3.4 Urinary studies

Patients were asked to void when they had a sensation of a full bladder to assess urinary flow rate in ml/s over time (s) using a digital urinary flow meter (Albany Medical SmartFlow), which recorded maximum flow (ml/s), time to maximum flow (s), void time (s) and voided volume (ml). Post-void residual volume was measured with bladder ultrasound scanner (Bardscan Realtime). Voided volume was divided by total bladder volume to calculate percentage voided. Examples of normal, flattened and intermittent flow profiles are shown in Figure 2.6.

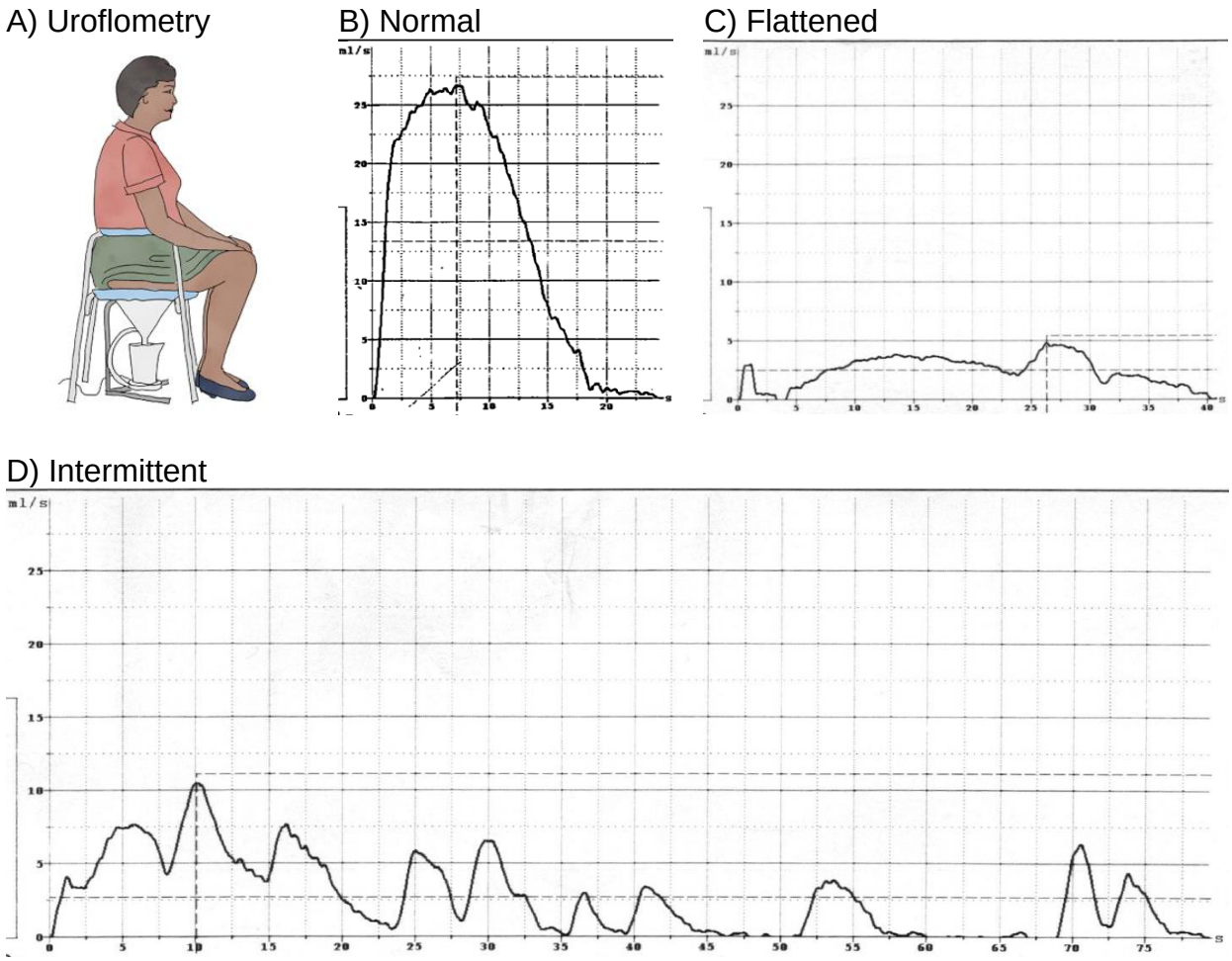


Figure 2.6. A) Patients were asked to void when they had a sensation of a full bladder. Examples of B) normal C) flattened and D) intermittent uroflow profiles. In a normal profile, there is a rapid rise and fall in flow rate. A flattened, prolonged profile is typically seen with outflow obstruction. An intermittent flow profile can be seen with abdominal straining to compensate for poor detrusor contractility.[62]

2.3.5 Dynamic sweat testing (DST)

DST was performed at the distal leg and forearm bilaterally (Figure 2.7).[63] After 1% pilocarpine iontophoresis at 2mA for 5 minutes, skin was coated with iodine and formation of sweat gland imprints on starch covered tape was recorded using a digital video camera (Sony Digital Camera HDR-CX240E). Pilocarpine directly stimulates M3 cholinergic receptors on sweat glands allowing assessment, by inference, of peripheral sweat gland innervation. Snapshots were taken when sweat droplets first became visible and when they started to merge. Image J 1.52i software was used to calculate the density of activated sweat glands/cm^3, sweat output/min/cm^3, and average sweat output/gland was calculated for each site and the mean value for both sides calculated.

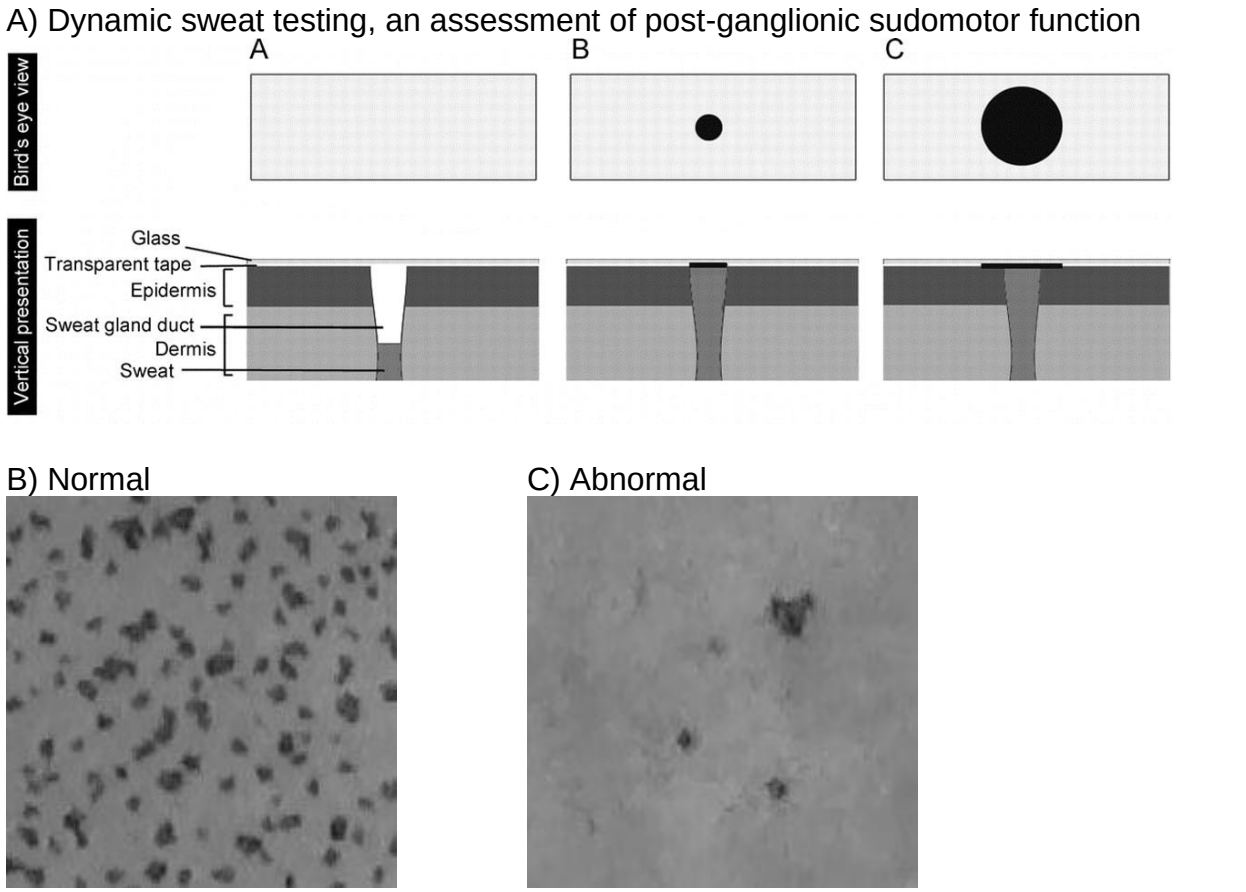


Figure 2.7. Schematic drawing demonstrating how DST was performed (A) with examples of normal (B) and abnormal (C) sweat production with very few sweat droplets visualised after pilocarpine iontophoresis. Part A reproduced from Provitera et al 2010, Neurology.[63]

2.3.6 Secretomotor testing

Lacrimal production was measured using Schirmer's test.[64] Strips of filter paper were folded 5mm from the end and placed in the lateral third of the lower palpebral fissure in each eye. After 5 minutes, the length of filter paper from the fold that had become wet was recorded in millimeters. The average value for both eyes was calculated.

Salivary production was measured using the unstimulated salivary production test.[65] Only water was permitted one hour prior to the salivary test. Patients were asked to swallow prior to the start of the test and then lean over and collect any saliva produced into a pre-weighed tube for 5 minutes. At the end of 5 minutes, they were asked to spit any saliva remaining in the mouth into the tube.

2.3.7 Patient reported outcomes

Patient reported outcomes were collected using the abbreviated and refined composite autonomic symptom score (COMPASS-31), the small fibre neuropathy symptom inventory questionnaire (SFN-SIQ), and the 36-item short form health survey (SF-36).

2.3.7.1 COMPASS-31

The COMPASS-31 is a concise validated tool which assesses six autonomic domains including orthostatic intolerance, vasomotor, secretomotor, gastrointestinal, bladder and pupillomotor symptoms with a recall period of between 1-5 years. The subscores are weighted and added together to give a total score of 100.[66] For repeat COMPASS-31 assessments following immune treatment, a recall period of 4 weeks was used.

2.3.7.2 SFN-SIQ

The SFN-SIQ assesses 12 sensory and autonomic symptoms including changes in sweating, diarrhea, constipation, micturition problems, dry eyes, dry mouth, orthostatic dizziness, palpitations, flushes, skin sensitivity, burning, restless legs, and sheet intolerance of legs, with patients asked to provide a score of 0 (never) to 3 (always) for each section.[67]

2.3.7.3 SF-36

The SF-36 is a 36-item short form developed to assess health status in clinical practice and research. It assesses eight health concepts, 1) physical limitations due to health problems, 2) social limitations due to physical or emotional problems, 3) role limitations due to physical health problems, 4) bodily pain, 5) general mental health (psychological distress and well-being), 6) role limitations due to emotional problems, 7) vitality (energy and fatigue), and 8) general health perceptions, with a scoring algorithm generating a possible range of 0 (worst possible health) to 100 (best possible health) for each of the eight domains.[68]

2.3.8 GAChR antibody testing

GAChR antibody testing was performed at the University of Oxford Neuroimmunology laboratory with a radioimmunoprecipitation assay using solubilised antigen from a human neuroblastoma (IMR-32) cell line bound to ^{125}I-epibatidine.[22] Samples testing positive (>100 pM) were then serially diluted and antibody concentrations expressed as pM (pmoles of ^{125}I-epibatidine precipitated per litre of serum).

2.3.9 Skin biopsies

2.3.9.1 Sample collection and storage

After intradermal injection of lidocaine, skin biopsies were collected using a 3-mm disposable punch under sterile technique (Figure 2.8). Samples were taken from the distal leg, 10 cm above the lateral malleolus, and distal thigh, 10cm above the patella. We collected 6-8mm samples, centred around a hair follicle, to maximise sampling of arrector pili muscles, sweat glands and artero-venous anastomoses. Samples were stored in in Zamboni's solution (2% paraformaldehyde, picric acid) for 4-6 hours then transferred to a polyvinylpyrrolidone cryoprotectant solution and stored at -20°C.

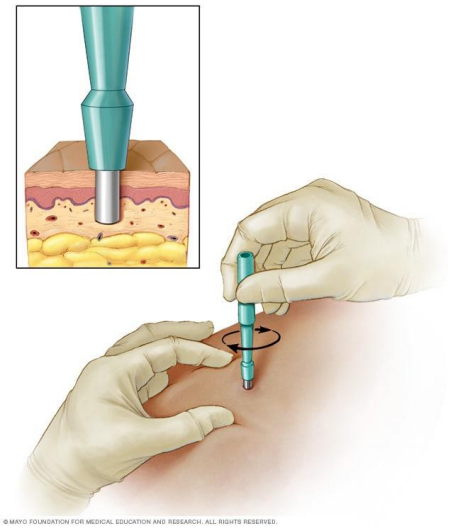


Figure 2.8. Punch skin biopsy collection technique. Figure reproduced from Mayo clinic, 2023, Skin biopsy [Online]. Available from URL https://www.mayoclinic.org/tests-procedures/skin-biopsy/about/pac-20384634 [Accessed 16th March 2023]

2.3.9.2 Preparation of free-floating sections

Samples were washed thrice with filtered phosphate-buffered saline (PBS) to remove cryoprotectant solution. A sliding freezing microtome (Leica 2000) was used to cut each sample into 50-µm-thick sections. Samples were embedded in Optimal Cutting

Temperature (OCT) medium and orientated with the epidermis perpendicular to the microtome blade. Any sections not used immediately were put into fresh cryoprotectant solution with an additional drop of Zamboni and stored at -20°C.

Free-floating sections were placed into PBS in 9 well glass plates for 15 minutes. After removal of PBS, 100µl of block solution was placed into each well. The glass plates were placed onto a shaking plate for 1-2 hours and stored overnight at -4°C.

2.3.9.3 Processing of sections for indirect immunofluorescence

After removal of block solution, solutions containing primary antibodies were placed into each well. For experiments using three different sets of primary and secondary antibodies, 33µl of each solution at 3x the intended concentration was placed into each well. Final antibody dilutions and sources are summarised in Table 2.4. The glass plates were placed onto a shaking plate for 1-2 hours and stored overnight at -4°C.

After removal of primary antibody solutions, wash solution was placed into each well for an hour and then removed. This was repeated three times, and solutions containing species-specific secondary antibodies coupled with cyanine 2, cyanine 3 and cyanine 5 fluorescent tags were placed into each well. Glass plates were protected from light and placed onto a shaking plate for 1-2 hours and stored overnight at -4°C.

2.3.9.4 Cleaning and mounting of sections

After three washes, PBS was placed into each well. Sections were inspected using a light microscope and any visible fragments or debris were removed. The plates were then stored overnight at -4°C.

Sections were mounted on a warmed platform attached to a light microscope. The PBS solution in each well was changed to remove any microfragments. Sections were transferred from each well onto a coverslip with warmed agar gel to remove any microfragments, and then onto warmed agar gel on a second coverslip. Sections were orientated so the epidermis was at the bottom of the coverslip. Excess agar gel was carefully removed from around each section. As the agar cooled and the sections were fixed in place, the coverslip was transferred to a ceramic holder.

The ceramic holders with the mounted coverslips were immersed in 95% alcohol for 30 minutes, 100% alcohol for 30 minutes, and then transferred to methylsalicylate solution for at least 30 minutes. The coverslips were then mounted onto glass slides with dibutylphthalate polystyrene xylene (DPX) and left to set at least overnight.

2.3.9.4 Primary and secondary antibodies

Collagen IV (COL IV) and ULEX Europaeus agglutinin 1 (ULEX) were used to visualise blood vessels and basement membranes, and PGP was used as a pan-neuronal marker. VIP and DβH were used as selective markers of cholinergic and noradrenergic fibres. A mixture of 3-well and 9-well experiments were to study the samples (Figure 2.9).

A) 9-well experiment

rTH-Cy2 mVIP-Cy3 Ulex-SA5	rPGP-Cy2 mpSyn64-Cy3 Ulex-SA5	rPGP-Cy2 mTAU-Cy3 Ulex-SA5
mVIP-Cy2 rDbH-Cy3 Ulex-SA5	mVIP-Cy2 rSubP-Cy3 Ulex-SA5	rS100-Cy2 mPGP-Cy3 Ulex-SA5
mCOLIV-Cy2 rPGP-Cy3 Ulex-SA5	rPGP-Cy2 mMBP-Cy3 Ulex-SA5	mVIP-Cy2 rCGRP-Cy3 Ulex-SA5

B) 3-well experiment

mCOLIV-Cy2 rPGP-Cy3 Ulex-SA5	mVIP-Cy2 rDbH-Cy3 Ulex-SA5	rDbH-Cy2 mVIP-Cy3 Ulex-SA5

Figure 2.9. Panel of primary and secondary antibody combinations used in experiments. For consistency, all quantification was performed on experiments using Cy3-coupled secondary antibodies, which emit a bright fluorescent signal (in bold).

Table 2.4. Antibody dilutions and sources

Marker (Abbreviation)	Dilution	Manufacturer
Primary antibodies		
Rabbit protein gene product 9.5 (rPGP)	1:400	Amsbio (Abingdon, UK)
Mouse protein gene product 9.5 (mPGP)	1:800	AbD Serotec (Kidlington, UK)
Rabbit vasoactive intestinal peptide (rVIP)	1:1000	Immunostar (Hudson, WI, US)
Mouse vasoactive intestinal peptide (mVIP)	1:300	Santa Cruz Biotech (Heidelberg, Germany)
Mouse collagen IV (mCOLIV)	1:800	Chemicon (Billerica, MA, USA)
Rabbit dopamine beta hydroxylase (rDβH)	1:150	Chemicon (Billerica, MA, USA)
Mouse Anti Phosphorylated α-Synuclein (mP-SYN)	1:6000	FUJIFILM Wako Pure Chemical Corporation (Japan)
Rabbit Calcitonin Gene Related Peptide (rCGRP)	1:1000	Immunostar (Hudson, WI, US)
Rabbit Substance P (rSub-P)	1:1000	Immunostar (Hudson, WI, US)
ULEX Europaeus agglutinin I (ULEX)	1:100	Vector Laboratories (Burlingame, CA, USA)
Secondary antibodies		
Rabbit Cyanine 2 (rCy2)	1:800	Jackson ImmunoResearch (Ely, UK)
Mouse Cyanine 2 (mCy2)	1:800	Jackson ImmunoResearch (Ely, UK)
Rabbit Cyanine 3 (rCy2)	1:1600	Jackson ImmunoResearch (Ely, UK)
Mouse Cyanine 3 (mCy2)	1:200	Jackson ImmunoResearch (Ely, UK)
Cy5-Streptavidin (SA5)	1:200	Jackson ImmunoResearch (Ely, UK)

2.3.9.5 Quantification of intra-epidermal fibres

An epifluorescence microscope (Axiocam 702 mono, Zeiss, Jena, Germany, EU) was used to study the slides. In accordance with international guidelines,[69] the number of fibres crossing the dermal-epidermal junction were counted, excluding branches within the epidermis, using a 20x objective. The length of epidermal surface was measured in mm, and the average number of fibres/mm for at least 3 sections was calculated.

2.3.9.6 Quantification of pilomotor fibres

An epifluorescence microscope (Axiocam 702 mono, Zeiss, Jena, Germany, EU) was used to study the slides to identify sections containing arrector pilorum muscles suitable for quantification.[39] Digital confocal images were acquired using a non-laser confocal system (Apotome2, Zeiss, Jena, Germany, EU) using a 20x objective. The single optical section with the most fibres running in focus for at least 100 µm parallel to the major axis of the muscle was selected from the Z-stack, ensuring the fibres were running in parallel to the focal plane with a tolerance of ±3.4°. A line perpendicular to the major axis of the muscle intercepting the most fibres was then traced and the width of the muscle measured. The average number of intercepts per muscle width for all muscles suitable for quantification in fibres/mm was calculated for each staining for each biopsy. For consistency, all quantification was performed on experiments using Cy3-coupled secondary antibodies, which emit a bright fluorescent signal.

2.3.9.7 P-syn score

Previous experiments with different dilutions of the mouse antibody recognising synuclein phosphorylated at 129 ser (Wako #015-25191) identified 1:6000 as the dilution with the best signal: background ratio,[48] and this dilution was used for the experiments in this

thesis. We searched for the presence of p-syn on nerves around autonomic structures (sweat glands, vessels, and arrector pili muscles) or running in the dermis as nerve fascicles or isolated fibres. P-syn colocalization with PGP was required to ensure we were observing neural p-syn deposits rather than non-specific staining. For each biopsy, we noted whether p-syn deposits were present (1) or absent (0) in the nerves in each of the 6 structures outlined in Figures 2.10, 2.11, and 2.12.

Structures analysed		
A. Blood vessels B. Sweat glands C. Arrector pili muscles	Autonomic p-syn subscore (0-3)	Total p-syn score (0-6)
D. Dermal nerve bundles E. Subepidermal neural plexus[1] F. Isolated fibres in upper dermis[2]		

Figure 2.10. Summary of structures used for calculating total p-syn score and autonomic p-syn subscore. [1]<200µm below basement membrane,[2]200- 500µm below basement membrane

For each biopsy, a total p-syn score was noted ranging from 0 (no deposits present) to 6 (at least one deposit in all 6 structures listed. An autonomic p-syn subscore was noted ranging from 0 to 3 (at least one deposit in all 3 cutaneous autonomic structures listed).

When bilateral samples were available, the average of both sides was calculated and used for further analyses.

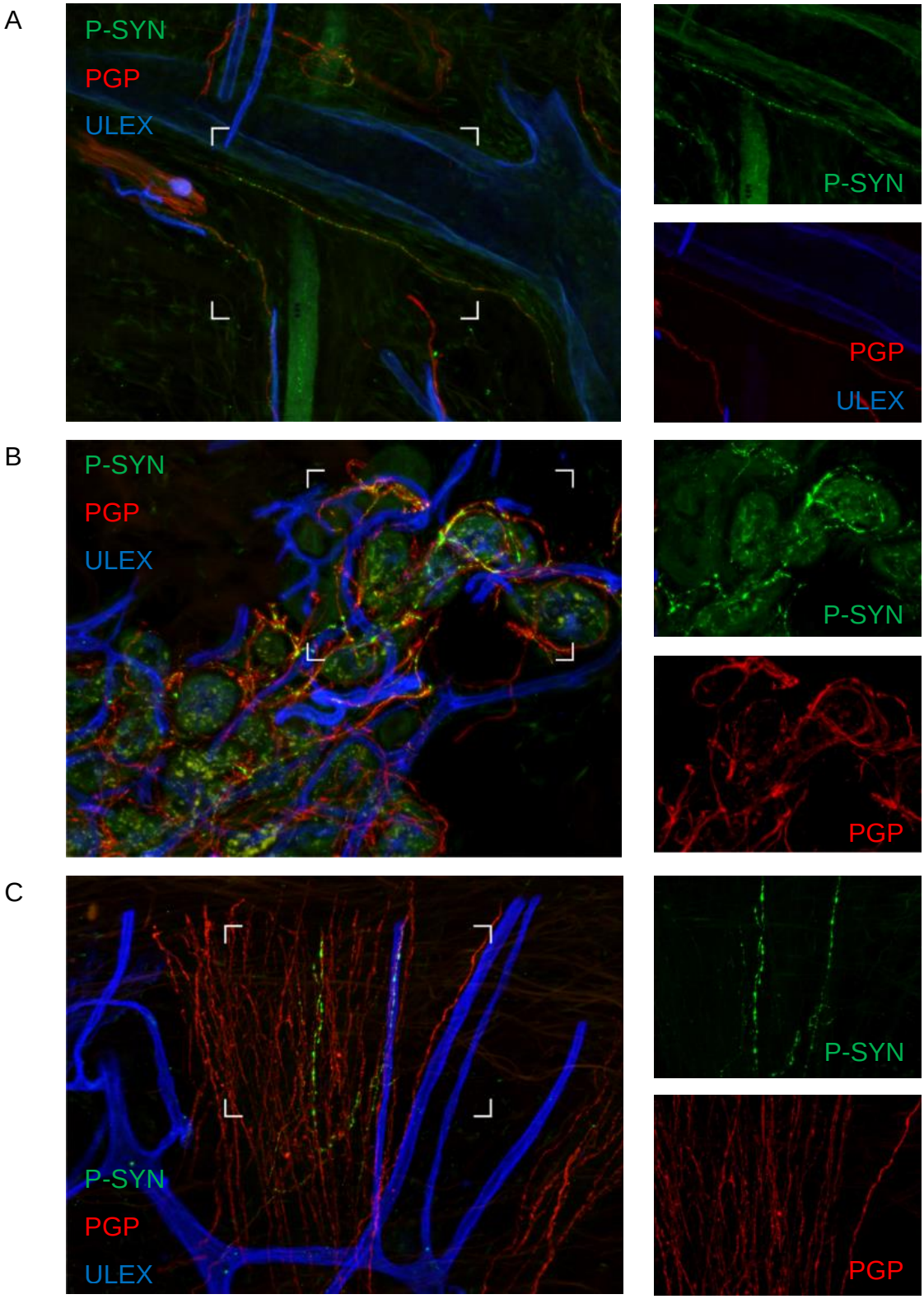


Figure 2.11. P-syn colocalising with pan-neuronal marker PGP in nerve fibres running along blood vessels (A), in sweat glands (B) and arrector pili muscles (C), adapted from Nolano et al (2022), Journal of Parkinsons disease.[48]

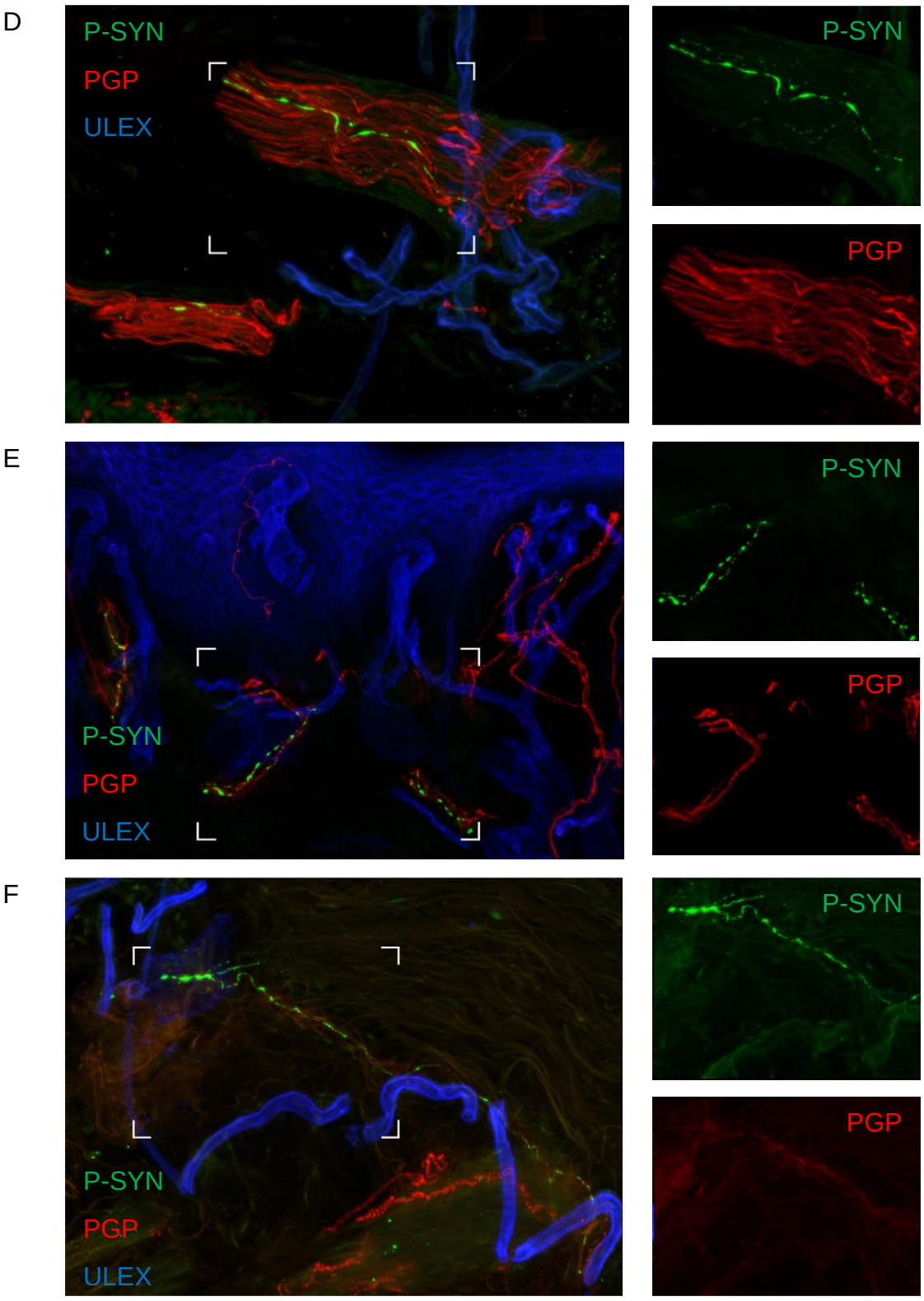


Figure 2.12. P-syn colocalising with pan-neuronal marker PGP in nerve fibres running in dermal nerve bundles (D), within the subepidermal plexuses (E) and running as isolated fibres in the upper dermis (F), adapted from Nolano et al (2022), Journal of Parkinsons disease.[48]

2.3.10 Statistical analysis

Data were captured electronically using a secure Research Electronic Data Capture (REDCap) platform. Statistical analysis was performed using R Studio, Version 1.2.1335. Summary data have been displayed as median, interquartile range for continuous data and numbers, percentages for categorical data. Distributions of data were assessed for normality by visual inspection and using Shapiro-Wilk tests. Pairwise comparisons were made with unpaired two-tailed T-tests/Wilcoxon rank-sum tests and group comparisons were made with ANOVA/Kruskal-Wallis tests with post-hoc comparisons using Tukey/Dunn's tests with Bonferroni corrections as appropriate. Chi-squared tests were used to compare categorical data. Spearman's rank correlation was used to assess the correlation between linear variables. $P<.05$ was considered significant.

2.3.10.1 Univariate and multivariate logistic regression models

Variables that were significantly different between groups were assessed in a univariate logistic regression model to determine if they were significant predictors of a particular diagnosis. Variables that were significant predictors at a univariate level were used in a multivariate model, after removing significantly correlated variables (defined as $\rho>0.40$ on Spearman's rank correlation). For correlated variables, the variable with the lowest *P*-value on univariate analysis was selected for use in multivariate analysis.

Chapter 3. Multimodal biomarkers in gAChR-positive AAG

3.1 Introduction

AAG is an uncommon but treatable disease typically presenting with subacute severe widespread autonomic failure. Patients develop disabling symptoms reflecting orthostatic hypotension, pupillary, gastrointestinal, genitourinary, sudomotor and secretomotor dysfunction. In 2000, Vernino et al reported fifty percent of patients presenting with subacute pandysautonomia had a detectable antibody to the ganglionic nicotinic acetylcholine receptor (gAChR) which mediates fast synaptic transmission at sympathetic, parasympathetic and enteric autonomic ganglia.[22] Passive and active immunisation studies have provided strong evidence for the pathogenicity of the ganglionic antibody.[24, 25] Previous experimental models have shown that the ganglionic antibody reversibly impairs synaptic transmission through internalisation of the ganglionic acetylcholine receptor, but the autonomic ganglia remain structurally intact.[23, 24] Studies in patients with AAG have shown postganglionic sudomotor dysfunction[70, 71] and pathological evidence of postganglionic autonomic and somatic nerve fibre loss on both sural nerve and punch skin biopsies, suggesting long-term immune attack against the autonomic ganglia may lead to post-ganglionic denervation.[72, 73]

Higher antibody levels have been associated with a greater degree of autonomic dysfunction,[74] while low titres <200pM are non-specific in the absence of autonomic failure.[32] Klein and Sandroni et al's studies in 2003-2004 in patients with idiopathic orthostatic hypotension reported high gAChR levels (>1000pM) were associated with a subacute onset, more severe dysautonomia and prominent cholinergic dysfunction, with sicca complex, abnormal pupillary light responses, lower gastrointestinal tract

dysfunction, and greater cardiovagal impairment on objective autonomic testing.[29, 30] Importantly, within the group of 18 patients with positive gAChR antibody levels >50pM, eight patients had an insidious symptom onset, without an antecedent event, and gradual progression. Within the chronic onset patients, half had high antibody titres (11600 ± 2080 pM) with severe cholinergic failure, and the other half with very low antibody levels (90 ± 10 pM) had no significant cholinergic symptoms. High antibody titres therefore corelated with greater autonomic dysfunction and more frequent cholinergic dysautonomia, rather than the temporal presentation of the illness alone, which had been historically used to classify patients presenting with autonomic failure. Therefore, the authors argued the clinical phenotype of autoimmune autonomic failure should be expanded to include more chronic presentations of autonomic failure, which may have previously been labelled as pure autonomic failure based on the insidious symptom onset and gradual disease progression.

At the Mayo Clinic, where large numbers of patients routinely undergo extensive panels of serological testing to investigate for possible autoimmune diseases, positive gAChR antibody levels have subsequently been reported in cases of postural tachycardia syndrome (POTS), chronic idiopathic anhidrosis, distal small fibre neuropathy, typically at low levels, without specific clinical characteristics differentiating them from seronegative cases, leading the authors to suggest there was no definite evidence that the gAChR antibody was pathogenic in these cases, but rather in keeping with an underlying autoimmune response.[75]

Therefore, in cases with low or negative gAChR antibodies, the decision to treat with immune therapy, which may be costly and associated with adverse effects, should be

considered on a case-by-case basis, taking into account the degree of suspicion of an autoimmune aetiology, including factors like an antecedent event, subacute onset, multiple organ/system involvement, or other autoimmune diseases, as well as the severity of autonomic failure and lack of response to trials of symptomatic pharmacological and non-pharmacological treatment. It is vital that clinicians offering trials of immune therapy perform standardised assessments before and after treatment to objectively quantify treatment effect.

Patients with AAG can respond to immunotherapy, but the response of individual patients varies, and multiple immunomodulatory agents may be needed.[26-28, 76] Previous studies have largely utilised cardiovascular and sudomotor assessments when attempting to objectively quantify the severity of autonomic failure and treatment response in patients with AAG, failing to capture the pupillary, urinary and secretomotor deficits that are prominent in this disease.[22, 27, 32, 76, 77] For example, Iodice and colleagues described a patient with low Composite Autonomic Severity Score (CASS), derived from cardiovascular and sudomotor testing, that poorly reflected her multiple prominent autonomic symptoms, measured by the COMPASS (Composite Autonomic Symptom Score) questionnaire, and a mismatch in CASS and COMPASS changes after immunotherapy.[27] We therefore investigated a cohort of thirteen individuals with gAChR-positive AAG with a multi-domain autonomic function testing protocol including cardiovascular, pupillary, urinary, sudomotor, lacrimal and salivary assessments to characterise their full clinical phenotype and repeated assessments following immunotherapy to try establish objective biomarkers to quantify treatment response that correlated with patient reported outcome measures. In addition to quantitative markers of

autonomic function, we performed immunofluorescence analyses on skin samples collected from one of the affected individuals to compare intraepidermal nerve fibre density and innervation of the autonomic adnexa before and after immune therapy.

3.2 Materials and methods

We studied patients referred to the Autonomic Unit at the National Hospital for Neurology and Neurosurgery, a national referral centre, with documented cardiovascular autonomic failure and elevated gAChR antibody levels >100pM identified from February 2005 to August 2019. Consent was obtained according to the Declaration of Helsinki. The study was approved by the local Research Ethics Committee and Health Research Authority.

3.2.1 GAChR Antibody Testing

GAChR antibody levels were measured by a radioimmunoprecipitation assay using solubilised antigen from a human neuroblastoma (IMR-32) cell line bound to ^{125}I-epibatidine as previously described,[22] and performed by the University of Oxford Neuroimmunology lab since 2005. Samples testing positive (>100 pM) were then serially diluted and antibody concentrations expressed as pM (pmoles of ^{125}I-epibatidine precipitated per litre of serum).

3.2.2 Quantitative Multi-domain Autonomic Testing (Figure 3.1)

Beat-to-beat measurements of blood pressure and heart rate were recorded and analysed with Labchart 8 Pro software (AD Instruments) as previously described.[55] Sympathetic function was evaluated by blood pressure response to the Valsalva manoeuvre, including the PRT[57, 78] and head-up tilt. Patients were passively tilted to 60° for up to 10 minutes. Tilt was terminated early if blood pressure fell significantly causing symptoms of cerebral hypoperfusion (e.g., loss of vision/hearing/reduced

responsiveness). OIR-tilt was calculated by dividing the change in systolic blood pressure in mmHg by the maximum time tolerated in minutes on tilt. If a patient tolerated a full 10 minutes of tilt, 10 was used as the denominator for the calculation. Parasympathetic function was assessed by HR_{DB} and Valsalva ratio. Blood samples were collected in the supine and tilted position for measurement of plasma noradrenaline using high performance liquid chromatography. Nerve conduction studies and EMGs were performed as part of routine clinical care.

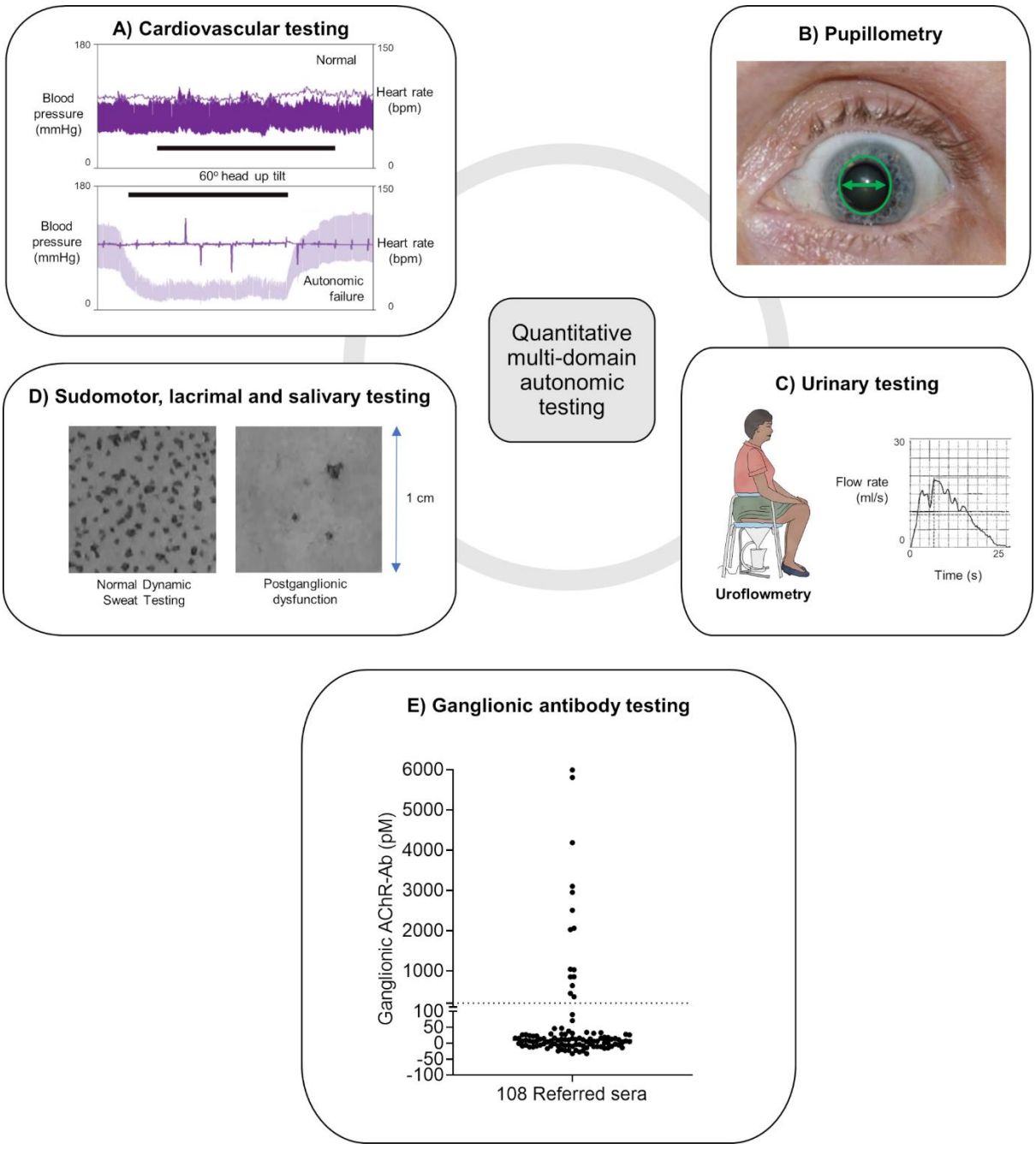


Figure 3.1. Quantitative multi-domain autonomic function testing and ganglionic antibody testing. Patients had A) cardiovascular testing, B) pupillometry, C) urinary, D) sudomotor, lacrimal and salivary testing and E) gAChR antibody testing performed before and after immune treatment. A) Head-up tilt to 60°. Top panel: stable blood pressure and heart rate profile in a healthy individual. Bottom panel: rapid blood pressure fall with no compensatory heart rate rise in a patient with autonomic failure. B) Pupil diameters were recorded with infrared pupillometry, capturing dark diameters and responses to light and pharmacological stimuli. C) Patients were asked to void into a uroflowmeter. Post-void residual volume was measured with ultrasound scan. D) DST. Left panel: even distribution of active sweat glands in a patient who received immunotherapy within 3 months. Right panel: reduced active sweat glands in a patient with AAG who first received immunotherapy 23 years after disease onset. Schirmer's test and the unstimulated salivary production test were used to measure lacrimal and salivary production (not illustrated). E) A radioimmunoprecipitation assay was used to test for gAChR antibodies. This graph illustrates gAChR antibody levels for 108 referred sera showing the 15 positive sera (all >200 pM, indicated by dotted line); 60 additional sera were reported <100 pM but not quantified further. (Part A adapted from Mathias 2006, with permission from Elsevier. [79]

Baseline pupillary dark diameters and responses to stimulation with white light and topical pharmacological agents were recorded with a custom built infrared pupillometer as previously described[60] or commercially available devices (Procyon P3000, Konan RAPDx, or Neuroptics DP2000). Impaired pupillary constriction to light and cholinergic supersensitivity (pupillary constriction with dilute 0.125% pilocarpine) indicated parasympathetic dysfunction. Delayed pupillary redilation following a light impulse to ¾ of baseline diameter, a lack of response to 4% cocaine, or adrenergic supersensitivity (abnormal dilation with 0.5% apraclonidine) indicated sympathetic dysfunction.[61] From May 2019, patients were also examined with a prolonged light stimulus to assess for pupillary fatigue, a unique phenomenon previously reported only in patients with seropositive autoimmune autonomic ganglionopathy.[31]

Urinary flow when voiding with the sensation of a full bladder was assessed by uroflowmetry (Albany Medical SmartFlow) and post-void residual volume measured using a bladder ultrasound scanner (Bardscan Realtime). Dynamic sweat testing was performed at both forearms and distal legs using 1% pilocarpine iontophoresis.[63] Pilocarpine directly stimulates M3 cholinergic receptors on sweat glands allowing assessment, by inference, of peripheral sweat gland innervation. Lacrimal production was measured using Schirmer's test and average for both eyes calculated.[64] Salivary production was measured using the unstimulated salivary production test.[65]

3.3.3 Questionnaires

The COMPASS-31 was used to assess orthostatic intolerance, vasomotor, secretomotor, gastrointestinal, urinary and pupillomotor symptoms.[66] The SF-36 was used to assess eight components affecting quality of life including physical functioning, role limitations

due to physical and emotional health, energy/fatigue, emotional well-being, social functioning, pain and general health.[68]

3.3.4 Longitudinal Assessments following Immunotherapy

Eleven patients received immunotherapy. From August 2018, patients were re-assessed with our full multi-domain autonomic testing protocol between 14-29 days following plasma exchange or IVIg, and between 4-6 weeks and three months after commencing corticosteroids. Prior to August 2018, testing was performed at various time frames as part of clinical care.

3.3.5 Skin Biopsy Analysis

Skin biopsies were obtained before and after immunotherapy from one of the patients. Specimens were fixed for 4-6 hours in Zamboni solution, cryoprotected in 20% sucrose in PBS and sent in a refrigerated package to the skin biopsy laboratory in Telese. Samples were cut into 50µm thick sections using a freezing slide microtome (Leica 2000R) and free-floating sections were processed for indirect immunofluorescence using antibodies to stain nerve fibres and vascular structures. Nerve fibres were marked with primary mouse and rabbit antibodies against the pan-neuronal marker PGP (AbD Serotec, 1:800: Biogenesis; 1:400), mouse antibody against myelin basic protein (MBP; Santa Cruz Biotechnology; 1:800, to assess myelinated fibres), mouse and rabbit antibodies against VIP (Santa Cruz Byotechnology, 1:300: Immunostar, 1:1000, to assess cholinergic fibres), and rabbit antibodies against DβH (Chemicon, 1:1000, to assess noradrenergic fibres). Vascular bed and basal membranes were marked with mouse antibody against COLIV (Chemicon; 1:800) and endothelia and epidermis were marked with ULEX. Species-specific secondary antibodies coupled with cyanine 2 and cyanine 3

fluorophores were used to visualise the structures of interest. Skin sections were mounted on coverslips with agar, dehydrated in alcohol, clarified in methyl salicylate and finally mounted in DPX. Digital images were acquired using a non-laser confocal microscope (Apotome; Zeiss).

3.3.6 Statistical Analysis

GraphPad Prism V.8 was used for statistical analysis. Data was tested for normality by the Shapiro-Wilk test. Summary data is provided as median (inter-quartile range) for simplicity as some data was not normally distributed. Baseline characteristics in idiopathic and paraneoplastic groups were compared with unpaired two-tailed t-tests/Mann-Whitney tests as appropriate. Baseline and follow up parameters after immunotherapy were compared with paired two-tailed t-tests/Wilcoxon signed-rank tests as appropriate. Spearman's rank/Pearson correlations were used to assess correlations between autonomic function testing and COMPASS-31 and SF-36 scores as appropriate. Two-sided $P<.05$ was considered significant.

3.4 Results

From February 2005 to August 2019, 168 patients with documented cardiovascular autonomic failure had blood samples sent to Oxford University for analysis of the ganglionic antibody. After subtraction of healthy control values, antibody levels ranged from -30 to 5990pM (Figure 3.1). Only fifteen patients had positive values >100pM (all >200pM). Two patients were excluded from this study due to concomitant diseases affecting the autonomic nervous system and the remaining thirteen included. By August 2018, five patients were deceased. For these patients, retrospective data was extracted from our patient databases and individual patient records and supplemented with direct

correspondence with local physicians. The remaining eight patients were prospectively recruited to undergo our full panel of autonomic testing and questionnaires at baseline and after treatment.

3.4.1 Clinical Presentation

Of the thirteen patients, seven (54%) were female. Median (IQR) age at onset was 54 (31-63) years. They all presented with pandysautonomia (Table 3.1). Eight (62%) had other autoimmune conditions including hypothyroidism (n=6), inflammatory bowel disease (n=3), psoriasis/eczema (n=3), Addison's disease (n=2), pernicious anaemia (n=2) and alopecia totalis (n=1). Four (31%) had antecedent infections and two (15%) had surgical procedures before developing autonomic symptoms (Table 3.2).

3.4.2 Autonomic Function Testing and Neurophysiology

All patients had widespread sympathetic and parasympathetic cardiovascular autonomic failure with orthostatic hypotension, reduced/absent HR_{DB}, and impaired heart rate and blood pressure responses to the Valsalva manoeuvre (Table 3.3). One patient had very low levels of supine noradrenaline (<100pg/ml), eight patients had low-normal levels (100-200pg/ml) and four had normal levels (200-500pg/ml), with no significant rise on tilt. None of the patients had a large fibre neuropathy. Five had absent sympathetic skin responses (four both upper / lower limbs, two lower limbs only), two had elevated thermal thresholds in both upper / lower limbs, and one had prolonged cutaneous silent periods in the lower limbs.

Twelve patients (92%) had impaired pupillary constriction to light; all five tested with dilute 0.125% pilocarpine showed cholinergic supersensitivity. All thirteen patients had evidence of bilateral sympathetic deficits; four (31%) had clinically apparent ptosis and all

eleven (100%) tested with 0.5% apraclonidine or 4% cocaine demonstrated bilateral Horner's syndrome. Three patients were tested with 1% hydroxyamphetamine: two patients tested within a year of disease onset showed normal pupillary dilation; the third, tested after 3 years of disease, had no response, indicating post-ganglionic sympathetic dysfunction. All seven patients (100%) examined with a prolonged 2-second bright light impulse demonstrated premature pupillary redilation during the light stimulus (Table 3.3).

Most patients (9/11; 82%) had urinary retention when first assessed and five (38%) required indwelling or intermittent catheterisation. Six out of eight patients (75%) who underwent uroflowmetry had an abnormal profile with prolonged void times, intermittent flow, and evidence of straining (Figure 3.2). The two patients with normal profiles had received immunotherapy prior to first uroflowmetry.

Table 3.1. Autonomic Symptoms and Signs in Autoimmune Autonomic Ganglionopathy

Age at onset/ sex	Orthostatic intolerance	Gastrointestinal	Genitourinary	Sudomotor	Secreto-motor	Pupillomotor
31 M	+	Gastroparesis	Retention ED Ejaculatory failure	Anhidrosis	Dry mouth	Ptosis, Photophobia
62 F	+	Constipation	Retention Recurrent UTIs	Anhidrosis	Dry eyes/ mouth	Photophobia
47 M	+	Constipation	Retention Recurrent UTIs ED Ejaculatory failure	Patchy anhidrosis	Dry mouth	Photophobia Unreactive pupils
46 F	+	Constipation Early satiety	Retention Recurrent UTIs	Anhidrosis	Dry eyes/ mouth	Photophobia Difficulty focusing
26 M	+	Gastroparesis	Retention ED Ejaculatory failure	Patchy anhidrosis	Dry eyes/ mouth	Dilated pupils Difficulty focusing
31 F	+	Constipation	Retention Recurrent UTIs	Anhidrosis	Dry eyes/ mouth	Photophobia Unreactive pupils
60 F	+	Constipation	Nocturia	Patchy anhidrosis	Dry eyes/ mouth	Photophobia R dilated pupil
21 F	+	Constipation	Voiding difficulties	Patchy anhidrosis	Dry eyes/ mouth	Photophobia
Retrospective cases						
63 F	+	Constipation Diarrhoea	Retention		Dry eyes/ mouth	L ptosis / meiosis
69 M	+	Constipation Diarrhoea	Retention ED		Dry eyes/ mouth	Photophobia Difficulty focusing
54 M	+	Constipation Vomiting	Retention ED	Hypo-hidrosis	Dry mouth	R ptosis Photophobia Unreactive pupils
55 M	+	Diarrhoea	Retention ED	Anhidrosis	Dry eyes/ mouth	
64 F	+	Constipation Diarrhoea	Nocturia		Dry mouth	Ptosis
n (%)	13 (100%)	13 (100%)	13 (100%)	10 (77%)	13 (100%)	10 (77%)

+, present; ED, erectile dysfunction; R/L, right/left; UTIs, urinary tract infections.

Table 3.2. GAChR Antibody Titres and Associated Clinical Features

Age at onset/sex	GAChR antibody, pM	Autoimmune conditions	Malignancy	Antecedent event
31 M	851	Eczema Crohn's disease Hypothyroidism Alopecia totalis Addison's disease		Colectomy
62 F	734			Urethral dilation
47 M	490	Psoriasis Ulcerative colitis		Respiratory infection
46 F	634	Hypothyroidism		Respiratory infection
26 M	5,805	Psoriasis Ulcerative colitis Hypothyroidism Addison's disease		
31 F	2,063		Ovarian teratoma, diagnosed during work-up for autonomic failure, 5 years after symptom onset	Respiratory infection
60 F	443	Hypothyroidism		Gastroenteritis
21 F	4,185			
63 F	2,953	Hypothyroidism	Rectal carcinoma, diagnosed within 1 year of symptom onset	
69 M	3,101		Chronic lymphocytic lymphoma, diagnosed within 2 years of symptom onset	
54 M	2,021		Rectal carcinoma, diagnosed 7 years after symptom onset	
55 M	856	Pernicious anaemia	Lung carcinoma, diagnosed 11 years after symptom onset	
64 F	5,990	Hypothyroidism Pernicious anaemia		
Number (%)		8 (62%)	3 (23%) diagnosed during work-up for autonomic failure 2 (15%) diagnosed >7 years after symptom onset	4 (31%)

Most patients (7/8; 88%) had impaired sweat production on dynamic sweat testing. The only patient with normal results was tested on maintenance immunotherapy, having first received immunotherapy within three months of disease onset. In 4/8 (50%) patients, sweat production was lower in the forearm compared to distal leg on one or both sides, in

keeping with a non-length dependent, ganglionic pathology. Most patients had reduced lacrimal production (9/11; 82%) and salivary production (6/8; 75%). The two patients with normal saliva production (>0.1gram/min) were on oral steroids when first tested.

Table 3.3. Cardiovascular Autonomic Testing and Pupillometry at Baseline

Age at onset/ sex	Disease duration at testing	Cardiovascular autonomic testing					Supine/ tilted NA, pg/ml	Pupillometry	
		Supine BP; HR	ΔBP; TT	OIR -tilt	HR_{DB}	VR		LR, R/L, %	Additional findings
31 M	4mo. [a]	101/61; 68	46/20; 45 s	61.3	0	1.04	362/ 382	20/19	0.5% apraclonidine: D 0.125% pilocarpine: C
62 F	9mo. [b]	151/67; 67	90/26; 1 min	9.0	4	1.01	163/ 176	18/15	Bilateral pupil fatigue 0.5% apraclonidine: D
47 M	1 yr.	139/77; 67	68/20; 4 min	17.0	4	1.12	172/ 173	17/17	Bilateral pupil fatigue 0.5% apraclonidine: D
46 F	2 yr.	140/74; 64	47/18; 4 min	11.8	5	1.06	191/ 194	14/11	Bilateral pupil fatigue 0.5% apraclonidine: D
26 M	2 yr.	114/77; 72	56/30; 5 min	11.6	1	1.00	181/ 203	8/6	Left pupil fatigue 0.5% apraclonidine: D 0.125% pilocarpine: C
31 F	5 yr.	146/101 ; 78	84/76; 3 min	28.0	5	1.00	108/ 105	4/4	Bilateral pupil fatigue 0.5% apraclonidine: D
60 F	8 yr.	177/87; 65	109/52; 6 min	18.2	2	1.08	135/ 137	10/28	Right pupil fatigue 0.5% apraclonidine: D
21 F	18 yr.	199/116 ; 75	142/83; 5 min	28.4	4	1.04	188/ 204	11/7	Left pupil fatigue 0.5% apraclonidine: D 0.125% pilocarpine: C
Retrospective cases									
63 F	4 wk.	146/82; 64	108/59; 3 min	25.3	4	1.02	149/ 176	33/32	Delayed T¾ 1% 4HA: D
69 M	1 yr.	164/94; 69	110/66; 5 min	22.0	0	1.00	229/ 246	2/3	4% cocaine: NR 1% 4HA: D
54 M	2 yr.	102/69; 67	47/30; 44 s	17.8	[c]	[c]	162/ 165	5/2	4% cocaine: NR 0.125% pilocarpine: C
55 M	2.5 yr.	154/95; 80	89/53; 5 min	17.8	3	1.00	238/ 277	0/0	4% cocaine: NR 1% 4HA: NR 0.125% pilocarpine: C
64 F	6 yr.	221/83; 56	163/28; 5 min	32.6	[d]	[d]	84/ 74	8/9	

4HA, 4-hydroxyamphetamine; ΔBP, change in systolic and diastolic blood pressure on 60-degree head up tilt; BP, blood pressure (mmHg); C, constriction; D, dilation; HR, heart rate (beats/min); NA, noradrenaline; NR, no response; LR, Light response; R/L, right/left; TT, time tolerated on tilt; VR, Valsalva ratio; wk./mo./yr., weeks/months/years; [a] This patient had received one 2 g/kg course of IVIg locally prior to referral to our unit, but had not noticed any symptomatic benefit. He had widespread autonomic failure on first autonomic assessment; [b] This patient had ganglionic antibody testing sent and treatment commenced locally (plasma exchange, intravenous methylprednisolone and oral prednisolone for 1 month) prior to testing. She reported significant symptomatic benefit from treatment but had still had evidence of widespread autonomic failure when first tested; [c] Cardiac arrhythmia during testing; HR_{DB} and VR could not be analysed; [d] Deep breathing and Valsalva manoeuvre poorly performed, HR_{DB} and VR could not be analysed.

3.4.3 Comparison of Paraneoplastic and Idiopathic AAG

Five patients (38%) were found to have malignancies including rectal carcinoma (n=2), lung carcinoma, ovarian teratoma and chronic lymphocytic lymphoma (n=1 each) (Table 3.2). Clinical features and autonomic testing at presentation did not distinguish between idiopathic and paraneoplastic AAG. Three patients had malignancies discovered during their initial investigations for autonomic failure. Two patients had unremarkable paraneoplastic screening on presentation and responded well to immunotherapy, but later deteriorated and were found to have malignancies after seven and eleven years respectively. Median duration of follow up was 8.5 years (IQR 6-12.5).

3.4.4 Immunotherapy Response and Complications of Treatment

One patient was found to have a locally advanced rectal carcinoma, deteriorated rapidly and died before planned treatment. Another historical patient treated symptomatically for pure autonomic failure was retrospectively found to have elevated gAChR antibodies after death. The other eleven patients had immunotherapy, with various treatments used depending on individual patient circumstances and treating physicians. The majority had plasma exchange (n=10). Other treatment included intravenous and/or oral corticosteroids (n=5), IVIg (n=3), rituximab (n=2), mycophenolate mofetil (n=5), azathioprine, methotrexate, and cyclophosphamide (n=1 each). Table 3.4 summarises the different treatments used, and effects noted by the patients and treating physicians.

Plasma exchange was associated with a rapid clinical improvement, beginning within days, with greater improvements seen in patients with early disease. Patients receiving plasma exchange within 2 years of disease onset reported improved symptoms across multiple domains, including orthostatic intolerance, gastrointestinal, bladder, sudomotor,

secretomotor and pupillomotor symptoms. In contrast, the patient with a 23-year history of symptoms before any immune treatment reported a much more limited and transient improvement in bladder function and dry mouth after initial plasma exchange treatment only. Of note, some patients developed significant complications after repeated exchanges. One patient had disrupted and incomplete treatment due to problematic vascular access after nine courses. Two patients developed deep vein thromboses after five and nine courses, the latter further complicated by a massive saddle pulmonary embolus requiring intra-arterial thrombolysis. One patient who initially received one course of IVIg (2g/kg) reported no immediate benefit but had a dramatic improvement with subsequent plasma exchange a month later. Two patients who improved with plasma exchange reported a less rapid but more sustained effect with regular IVIg; one patient developed a mild rash managed with topical steroid treatment and switching IVIg brand. Three patients treated with high dose oral prednisolone (1mg/kg) had striking improvements in multiple domains. One had intolerable adverse effects with weight gain, hair loss, low mood and anxiety, and the other had supine hypertension, which both improved with dose reduction. Four patients commenced on mycophenolate mofetil for maintenance immunotherapy had stabilisation of disease with no major adverse effects; one patient had to discontinue treatment due to anaemia. One patient started on azathioprine developed drug-induced hepatitis, was switched to methotrexate and remained well for five years without other treatment.

3.4.5 Quantitative Autonomic Biomarkers to Monitor Treatment Response and Correlations with Patient Reported Outcome Measures

Following immunotherapy, there were significant improvements in ΔSBP (56 [49-94] mmHg to 46 [14-71] mmHg , *P*=.03) and time tolerated on head-up tilt (2 [1-5] minutes to 10 [10-10] minutes , *P*=.006), OIR-tilt (33.3 [17.8-61.3] to 5.2 [1.4-8.2], *P*=.007), HR_{DB} (1.5 [0.0-3.3] bpm to 4.5 [3.0-6.3] bpm, *P*=.02) pupillary constriction to light (12.0 [5.5-18.0] % to 19.0 [10.6-23.8]%, *P*=.02), saliva production (0.01 [0.01-0.05] g/min to 0.08 [0.02-0.20] g/min, *P*=.03). Total COMPASS-31 autonomic symptom scores improved significantly (52 [34-64] to 17 [8-31], *P*=.03), as well as secretomotor and pupillomotor subscores. There were improvements in Valsalva ratio (1.02 [1.00-1.05] to 1.12 [1.00-1.23], *P*=.05 and PRT (28.3 [25.4-31.9] to 21.8 [2.6-29.4], *P*=.07), although statistical significance was not reached at the .05 level. Supine noradrenaline levels, Δ NA on tilt, uroflowmetry parameters, post-void residual volume, sweat production, lacrimal production, and SF-36 subscores were not significantly different in the cohort, although individual patients did show improvements (Table 3.5).

At baseline, the OIR-tilt correlated with COMPASS-31 total scores (r= 0.841, *P* =.01), COMPASS-31 orthostatic tolerance subscores (ρ= 0.792, *P*=.03), SF-36 physical function subscores (r =-0.716, *P* =.046) and SF-36 role limitations due to physical health (ρ=-0.784, *P* =.048). Following treatment, improvements in the OIR-tilt correlated with changes in total COMPASS-31 scores (r=0.889, *P*=.02) and orthostatic tolerance subscores (r=0.896, *P*=.02).

Table 3.4. Immunotherapy Response and Complications of Treatment

Age at onset /sex	Disease duration	Immunotherapy	Treatment effect and complications
31	3 mo.	IVIg 2 g/kg	No immediate effect
M	4 mo.	PLEX[5] × 3 (6 mo.)	Dramatic improvement orthostatic tolerance, ptosis,
	2 yr.	PLEX[5] × 2 (6 mo.), Azathioprine (2 wk.), Methotrexate (5 yr.)	stopped catheterization. Resumed full-time employment, playing rugby. Hair returned (alopecia totalis). Hepatitis (azathioprine).
62F	9 mo.	PLEX[5], IVMP[3], PO Pred 60-35 mg OD (tapered over 4 mo.)	Improved orthostatic tolerance, constipation, sweating, urinary, sicca symptoms. Weight gain (steroids).
47	2 yr.	PLEX[5] × 3 (6 mo.)	Improved orthostatic tolerance, sweating, bowels,
M	5 yr.	PLEX[5] × 3 (6 mo.)	urinary symptoms, light tolerance.
46F	2 yr.	PLEX[5] × 9 (2.5 yr.)	Improved orthostatic tolerance, bowels, urinary, sicca symptoms. DVT/PE (9th PLEX).
	5 yr.	Pred 60–25 mg (tapered over 3 mo.) [a]	More dramatic, sustained improvement in orthostatic tolerance, sweating, bowels, urinary, sicca symptoms. Weight gain, hair loss, low mood, anxiety (steroids).
31F	5 yr.	Resection ovarian teratoma	Improvement in sweating and salivation.
	6 yr.	PLEX × 9 (4 yr.)	Improved orthostatic tolerance, urinary and bowel symptoms. Vascular access difficulties (PLEX).
	10 yr.	IVIg 2 g/kg × 4 (6 mo.) [a]	Improved orthostatic tolerance, constipation, urinary symptoms, dry eyes, sweating. Rash (IVIg).
60F	7 yr.	PLEX[5] × 3 (6 mo.) [b]	Improved constipation after 2nd PLEX.
26	5 to	Spontaneous gradual recovery orthostatic tolerance, urinary retention, sweating, sicca	
M	20 yr.	symptoms, photophobia. Stopped intermittent catheterization.	
	22 yr.	40 mg HCT/day (3 yr.) [c]	Improved orthostatic tolerance, bowels, weight stab.
21F	23 yr.	PLEX[5]	Transient improvement urinary symptoms, dry mouth
	33 yr.	PLEX[5] × 4 (9 mo.)	Transient improvement urinary symptoms, dry mouth, orthostatic tolerance after 5th PLEX. DVT (5th PLEX).
	34 yr.	PO Pred 50–30 mg OD (tapered over 3 mo.) [a]	Dramatic improvement orthostatic tolerance, photophobia, urinary, sicca symptoms. Supine hypertension (steroids).
Retrospective cases			
6	2 yr.	Chlorambucil, Pred × 3,	Improved lymphadenopathy, no effect autonomic
9		Rituximab × 4 for CLL	symptoms.
M	3 yr.	PLEX[5] × 3 (1 yr.)	No improvement, found to have progression of CLL.
	5 yr.	Cyclophosphamide, Fludarabine × 6 (5 mo.)	No improvement in chronic constipation, dry mouth, suprapubic catheter, wheelchair dependence.
5	3 yr.	PLEX[5] × 3 (9 mo.)	Improved orthostatic tolerance, sweating.
4	7 yr.	Deterioration in symptoms. Found to have locally advanced rectal carcinoma. Declined	
M		rapidly and died after 4 mo.	
5	3 yr.	PLEX[5] × 4 (6 mo.), MMF 1.5mg	Improved orthostatic tolerance, erectile dysfunction
5		BD, IVIg 2g/kg every 3 mo.	dry mouth, micturition, sweating
M		(4 yr.)	
	7 yr.	PLEX[5]/IVIG every 6 wk. × 3 (1 yr.)	No improvement
	8 yr.	Rituximab × 2	No improvement
	11 yr.	Deterioration in symptoms, weight loss, voice change; found to have recurrent laryngeal nerve palsy due to metastatic lung adenocarcinoma, declined rapidly, died after 1 yr.	

CLL, chronic lymphocytic lymphoma; DVT, deep vein thrombosis; HCT, hydrocortisone; IVIg, intravenous immunoglobulin; IVMP[3], intravenous methylprednisolone (3 days); MMF, mycophenolate mofetil; PE, pulmonary embolus; PLEX[5], plasma exchange (5 days); PO Pred, oral prednisolone; [a] These 4 patients have subsequently had introduction of mycophenolate mofetil (1–1.5g BD) with stabilization of disease; [b] This patient developed anaemia after mycophenolate and treatment had to be discontinued; [c] Commenced for subsequent diagnosis of Addison's disease.

Table 3.5. Comparison of Assessments Before and After Immunotherapy

Parameter (n)	Median (IQR)		Mean change (95% CI)	*P* value
	Baseline	Follow up		
gAChR antibody levels, pM (11)	837 (506–2021)	427 (220–1068)	−709 (−1874 to 456)	0.08
Quantitative autonomic biomarkers				
Supine SBP, mmHg (11)	117 (102–155)	133 (120–160)	4.6 (−14.0 to 23.3)	0.59
Δ SBP on tilt, mmHg (11)	56 (49–94)	46 (14–71)	−24 (−41 to −7)	0.03*
Time tolerated on tilt, minutes (11)	2 (1–5)	10 (10–10)	5.6 (2.9 to 8.4)	0.006**
OIR-tilt (11)	33.3 (17.8–61.3)	5.2 (1.4–8.2)	−32.3 (−52.4 to −12.3)	0.007**
HR_{DB} (10)	1.5 (0.0–3.3)	4.5 (3.0–6.3)	2.9 (0.8 to 5.0)	0.02*
Valsalva ratio (10)	1.02 (1.00–1.05)	1.12 (1.00–1.23)	0.14 (−0.02 to 0.31)	0.05
Pressure recovery time, seconds (6)	28.3 (25.4–31.9)	21.8 (3.6–29.4)	−9.5 (−20.6 to 1.5)	0.07
Δ Noradrenaline on tilt, pg/ml (5)	17.0 (8.0–30.5)	17.0 (7.0–55.5)	14 (−33.0 to 53.0)	0.6
Pupillary light response, % (9)	12 (6–18)	19 (11–24)	6 (1 to 11)	0.02*
Post-void residual volume, ml (7)	280 (84–413)	51 (0–127)	−172 (−383 to 38)	0.16
Uroflowmetry (5)				
Maximum flow, ml/s	26 (13–34)	19 (11–30)	−4 (−12 to 4)	0.27
Time to max flow, s	10 (5–14)	8 (7–10)	−1 (−9 to 7)	0.74
Void time, s	79 (48–204)	68 (39–75)	−9 (−50 to 33)	0.81
Volume voided, ml	279 (241–542)	318 (219–616)	−9 (−37 to 113)	0.63
Percentage voided, %	73 (51–88)	83 (64–100)	12 (−15 to 39)	0.38
Sweat output, nL/cm^2/min (6)				
Forearm	143 (18–350)	185 (68–225)	−27 (−219 to 164)	0.73
Distal leg	50 (5–121)	37 (27–171)	27 (−32 to 87)	0.31
Average	94 (19–269)	111 (54–189)	0 (−100 to 100)	1.00
Tear production, mm (6)	5 (0–18)	4 (1–12)	−3 (−11 to 5.5)	0.45
Saliva, g/min (6)	0.01 (0.01–0.05)	0.08 (0.02–0.20)	0.07 (−0.01 to 0.16)	0.03*
Questionnaires				
COMPASS-31 total (6)	52 (34–64)	17 (8–31)	−28 (−51 to −5)	0.03*
Orthostatic intolerance	32 (19–32)	6 (0–18)	−16 (−31 to −1)	0.09
Vasomotor	0.0 (0.0–0.0)	(0.0–0.0)		
Secretomotor	8.6 (1.6–11.3)	2.1 (0.0–4.8)	−4.6 (−8.5 to −0.8)	0.03*
Gastrointestinal	9.8 (6.0–12.3)	5.8 (1.8–7.8)	−4.5 (−11.2 to 2.3)	0.15
Bladder	1.7 (0.0–5.8)	0.0 (0.0–3.1)	−1.3 (−4.1 to 1.6)	0.38
Pupil	2.3 (1.3–3.4)	1.7 (0.5–2.5)	−0.9 (−1.8 to −0.1)	0.03*
SF-36 (6)				
Physical functioning	20 (9–54)	45 (8–66)	13 (−32 to 58)	0.51
Role limitations (P)	0 (0–25)	0 (0–63)	8 (−61 to 78)	1.00
Role limitations (E)	67 (0–100)	100 (0–100)	11 (−17 to 40)	1.00
Energy/fatigue	30 (13–55)	53 (48–59)	22 (−6 to 49)	0.10
Emotional well-being	32 (28–46)	44 (32–76)	15 (−3 to 33)	0.25
Social functioning	50 (13–78)	63 (28–91)	10 (−32 to 53)	0.81
Pain	45 (10–90)	55 (19–81)	3 (−40 to 46)	0.89
General health	35 (9–56)	50 (36–60)	14 (−7 to 35)	0.14

*ΔSBP, change in systolic blood pressure; COMPASS-31, Composite Autonomic Symptom Score; Role limitations (E, emotional; P, Physical); SBP, systolic blood pressure; SF-36, Short Form Health Survey-36; * P value < 0.05; ** P value < 0.01.*

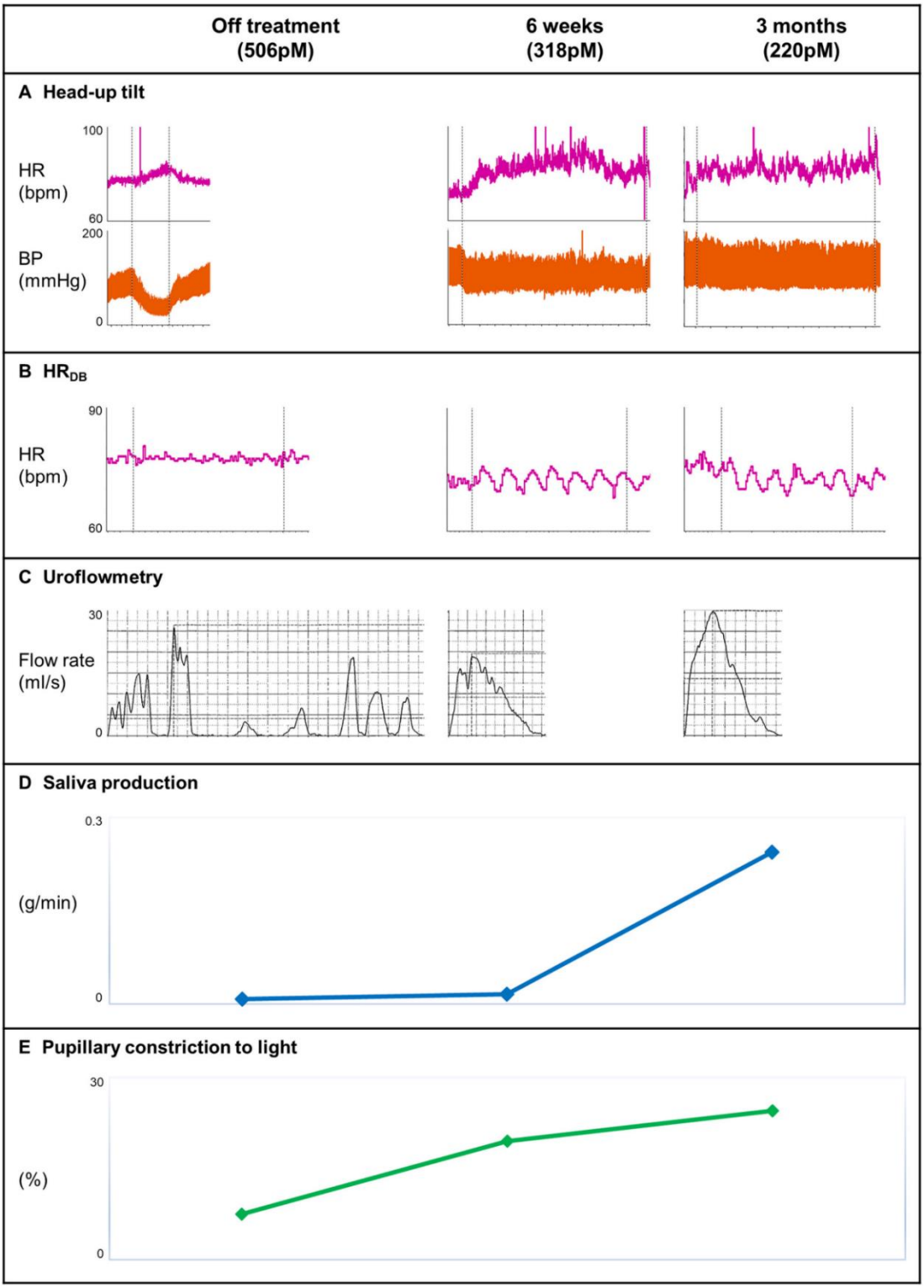


Figure 3.2. Serial multi-domain autonomic testing illustrating response to high dose oral prednisolone therapy. Before treatment (gAChR antibody level 506pM), blood pressure (BP) fell rapidly with head-up tilt (A), which had to be terminated after 1 minute. Three months after oral prednisolone (gAChR antibody level 220pM), she tolerated a full 10 minute tilt without a fall in blood pressure. There was a marked increase in HR_{DB} (B). Dotted vertical lines in (A) and (B) indicate when tilt and deep breathing started and stopped. Uroflowmetry profile (C) before treatment was abnormal with a prolonged void time and intermittent flow. After treatment, urine flow was smooth and void time reduced. Saliva production (D) and pupillary constriction to light (E) both increased greatly after treatment.

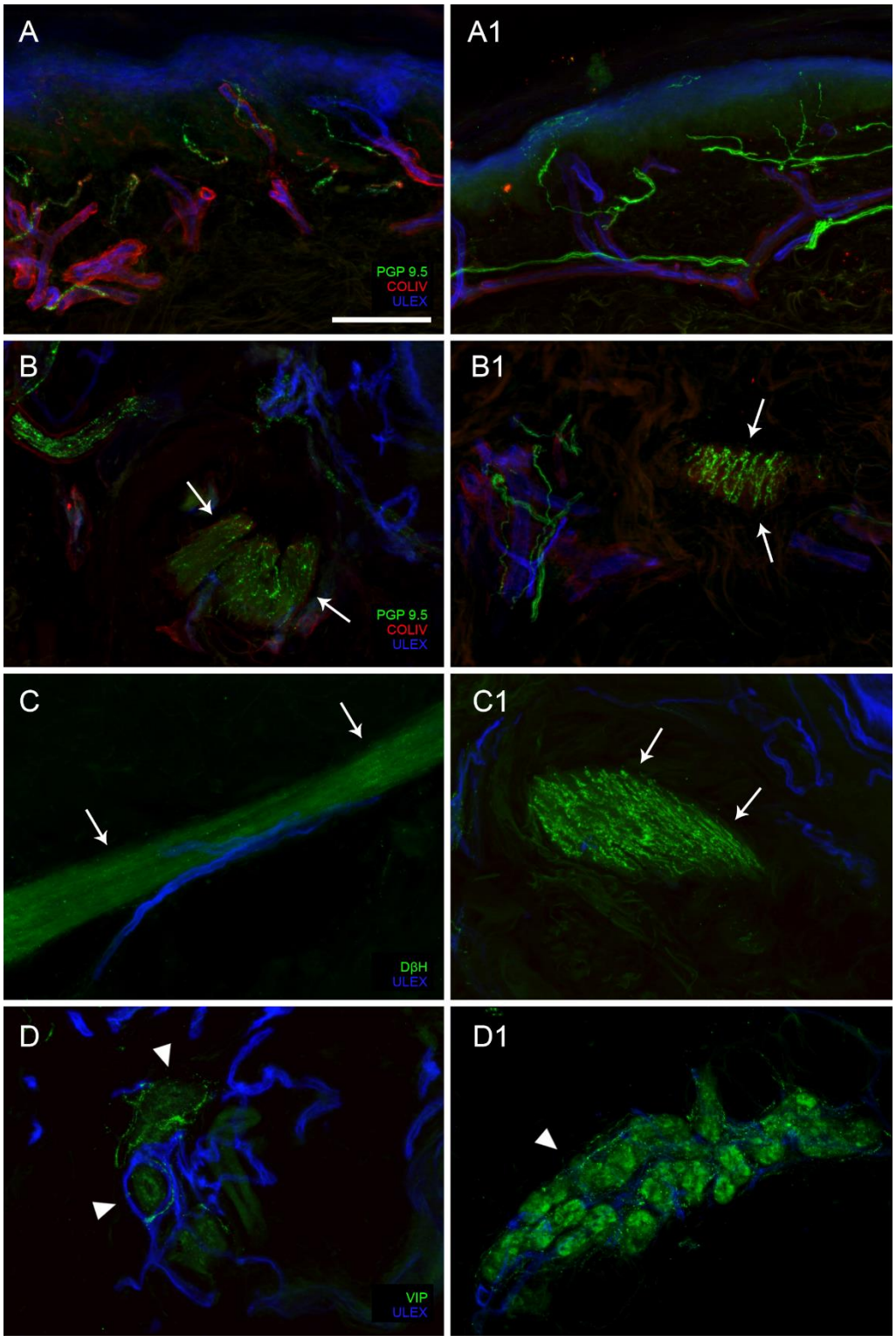


Figure 3.3. Skin biopsies with severe denervation (A-D) and improvement following immunotherapy (A1-D1). Initial biopsies demonstrated a highly abnormal and fragmented staining pattern, poor subepidermal neural plexus and lack of intraepidermal fibres (A). A severe loss of pilomotor fibres was evident using pan-neuronal marker PGP (B) and a complete denervation with noradrenergic marker DβH (C). A severe loss of VIP-ir cholinergic fibres around a sweat gland was evident (D). After treatment, there was marked improvement in fibre density and morphology (A1- D1). Arrows in B, B1, C, C1: arrector pili muscles. Arrowheads in D and D1: sweat glands. Scale bar = 100μm.

3.4.6 Illustrative Case

A 21-year-old woman presented in the 1980s with subacute pandysautonomia. Two decades later, after the discovery of the ganglionic antibody by Vernino et al,[22] historic banked frozen plasma was sent to Oxford University for gAChR antibody testing and high levels were found (4185pM). Initial plasma exchange treatment resulted in transient changes in urinary and salivary symptoms only and she declined further immunotherapy. Ten years later, she represented with worsening symptoms. She required a mobility scooter due to severe orthostatic intolerance, dentures following dental caries caused by xerostomia, digital stimulation for bowel evacuation and experienced frequent pre-syncopal symptoms when straining to void her bladder. Quantitative multi-domain autonomic testing demonstrated widespread autonomic failure. As before, plasma exchange initially produced improvements in salivary and urinary function only, but after four courses, a return of reflex tachycardia on tilt and Valsalva manoeuvre was observed. After a fifth course, she had a modest transient improvement in orthostatic tolerance but unfortunately developed a deep vein thrombosis treated with 3 months of anticoagulation. We then started high dose oral prednisolone (1mg/kg) and she reported marked symptomatic improvement in orthostatic tolerance, urinary, lacrimal, salivary and pupillomotor symptoms within weeks. Repeat testing at six weeks and three months demonstrated remarkable sustained improvements in OIR-tilt (51 to 0.4), HR_{DB} (0 to 6), pupillary light response (8 to 25% constriction), uroflowmetry profile, saliva production (0.01 to 0.24g/min), average sweat production (7 to 121nL/cm^2/min), total COMPASS-31 scores (50 to 10), SF-36 physical function scores (10 to 100) and gAChR antibody levels (506 to 220pM) (Figure 3.2). She subsequently remained well on a tapering prednisolone

regimen with maintenance mycophenolate mofetil with no orthostatic symptoms reported at her latest review 12 months later.

Punch skin biopsies were collected from the left forearm, left thigh and both distal legs prior to her 3rd course of plasma exchange in August 2018, 32 years after disease onset. There was a complete loss of intra-epidermal nerve fibres in all sites sampled, with a very poor subepidermal neural plexus (Figure 3.3). Innervation of the dermal adnexa including the arrector pili muscles, sweat glands, and blood vessels was also markedly reduced. All visualised PGP-immunoreactive fibres showed a strikingly abnormal, fragmented pattern, suggesting ongoing active degeneration in the remaining nerves. There were few fragments of VIP-ir cholinergic and DβH-ir noradrenergic fibres. Follow up samples were collected from the left thigh and distal leg, a few millimetres from the original biopsy sites, in November 2019, 6 months after commencing oral prednisolone therapy. These showed a clear improvement in cutaneous innervation with several new fibres in the subepidermal neural plexus and few fibres reaching the epidermis. Intra-epidermal nerve fibre density was still below 5% cut-off for age and sex but improved from 0.2/mm in the leg and 0.3/mm in the thigh to 3.9/mm and 4.1/mm.[80] In addition, the morphology of the visualised PGP-ir nerve fibres appeared improved, with resolution of the previously observed fragmentation and derangement of the neural network around the autonomic adnexa. Sudomotor, pilomotor, and vasomotor innervation assessed on sections immunostained with the pan-neuronal marker PGP and selective cholinergic and noradrenergic markers VIP and DβH by a semiquantitative method (4: normal innervation, 3: mild loss of fibres, 2: severe loss of fibres, 1: rare surviving fibres) showed a mean density of 2.5 compared to 1 at the first assessment.

3.5 Discussion

We present the most comprehensively phenotyped cohort of patients with seropositive AAG and the largest longitudinal series studying immunotherapy effect to date. Our novel multi-domain autonomic function testing protocol allowed us to comprehensively quantify the breadth of autonomic deficits in patients with AAG and identified key biomarkers measuring cardiovascular, pupillary and salivary function that improved significantly following immunotherapy. Immunotherapy can be associated with significant and potentially life-threatening complications, so quantitative biomarkers are important to objectively assess the effect of immune therapy and guide clinical decision making. Assessing multiple domains increases the sensitivity of detecting early changes with immune treatment, which may reflect potential for more widespread recovery. In our illustrative case, cardiovascular and sudomotor testing was unchanged with initial plasma exchange, but reproducible improvements in salivary and urinary domains suggested potential reversibility despite her prolonged history. Introduction of high dose oral prednisolone followed by maintenance mycophenolate mofetil resulted in remarkable widespread improvements on autonomic function testing, patient reported outcome measures, and cutaneous innervation. The case highlights that even in longstanding disease, with significant abnormalities of postganglionic innervation, AAG is potentially treatable with adequate immunotherapy.

Vernino and Lennon et al previously described an experimental animal models of AAG.[23-25] Rabbits immunised with a recombinant gAChR subunit fusion protein who developed high gAChR antibody levels had reduced neurons in the myenteric plexus ganglia and internalisation of synaptic gAChR, but no evidence of tissue inflammation. Mice injected

with serum from the immunised rabbits and patients with AAG had impaired cholinergic synaptic transmission, which was reversible, again suggesting antibody action was via internalization and accelerated degradation of the synaptic gAChR, rather than cytotoxic destruction, as seen in experimental autoimmune myasthenia gravis. These experimental models provide evidence that AAG is reversible in its early stages. With longstanding disease, there may be a degree of postganglionic neuronal loss that is irreversible in some patients,[72] but potential improvement in some with ongoing immune attack, as in our illustrative case. We would advocate taking a similar approach to other autoimmune neuropathies, such as CIDP, where international consensus guidance recommends treatment decisions should consider whether there is active disease as evidenced by progression, relapse, or persistent treatment dependence, or fixed deficits that cannot improve due to severe chronic axonal degeneration.[81]

The striking fragmented pattern of the visualised epidermal and dermal nerve fibres on the baseline skin biopsies has been seen in a previous case report on another patient with longstanding seropositive AAG,[72] who had a limited response to initial immunotherapy (a trial of IVIg) and declined further immune treatment. Larger studies examining skin biopsies in AAG are not currently available, but a study in demyelinating Guillain-Barre syndrome (GBS) found 11/20 (55%) patients had abnormal cutaneous innervation at the distal leg 27.0 ± 5.7 days after symptom onset, with reduced epidermal innervation, fragmented subepidermal nerve plexuses and a beaded appearance of dermal nerves suggesting active nerve degeneration.[82] Longitudinal skin biopsy studies suggest interventions in patients with impaired glucose tolerance can be associated with evidence of cutaneous axonal regeneration,[35] but this was uncommon in patients with

longstanding diabetes (mean duration 29 ± 9 years), where most individuals had reduced or absent subepidermal plexuses from which intra-epidermal nerve fibres would be expected to regrow.[83] In AAG, the presence of reduced numbers of morphologically abnormal, apparently fragmented dermal nerves, rather than complete denervation, may indicate potential for recovery even with prolonged disease.

While our patients all presented with pandysautonomia, they commonly reported orthostatic intolerance was their most disabling autonomic symptom. Prior to treatment, all patients had marked symptomatic falls in blood pressure on tilt necessitating rapid return to a supine position. After immunotherapy, the fall in blood pressure decreased and time tolerated on tilt increased. Incorporating both these variables, the orthostatic intolerance ratio improved significantly following treatment. The orthostatic intolerance ratio correlated with the severity of orthostatic intolerance and total autonomic symptoms, and physical limitations reported by patients at baseline. Furthermore, following immune treatment, improvements in orthostatic intolerance ratio correlated with improvements in patient reported autonomic symptoms, suggesting it is a responsive and relevant biomarker and potentially useful outcome measure in future treatment trials.

Our multi-domain testing protocol revealed several other clinically important insights relevant to the management of patients with AAG. Prior to immunotherapy, patients commonly had urinary retention and abnormal uroflowmetry profiles reflecting voiding dysfunction and evidence of straining. Straining should be discouraged as it can cause precipitous falls in blood pressure by reducing venous return and may also lead to upper urinary tract damage. Urinary retention increases risk of infections that may exacerbate autonomic failure symptoms. Catheterisation should be considered to ensure regular

emptying if post-void residual volumes are persistently >100ml. All our patients demonstrated pupil fatigue, premature redilation during a prolonged light impulse, which appears to be a unique phenomenon only seen in patients with gAChR-positive AAG.[31, 33] Only four (31%) of our cohort had clinically apparent ptosis, but all 11 (100%) who underwent pharmacological pupillary testing demonstrated subclinical bilateral sympathetic deficits. Symmetrical sympathetic pupillary deficits can be difficult to detect clinically, especially if there are co-existing parasympathetic deficits.[61] With a quantitative pupillometry protocol utilising physiological and pharmacological stimuli, we identified sympathetic and parasympathetic deficits in 12/13 (93%) patients. The profound impairments across cardiovascular, pupillary, urinary, sudomotor and secretomotor domains consistently documented in our patient cohort appears to be a phenotypic signature for AAG patients with high titres of ganglionic antibodies. The characteristic phenotype of cholinergic autonomic failure with orthostatic hypotension and other manifestations of sympathetic failure is consistent with the underlying pathophysiology of antibody-mediated impairment of synaptic transmission at sympathetic and parasympathetic autonomic ganglia.[25, 84, 85] We found preserved postganglionic pupillary function in assessments performed early in the disease course and normal dynamic sweat testing in a patient who had received prompt immunotherapy, suggesting earlier immunotherapy may help reduce postganglionic denervation. We advocate initiating induction immunotherapy regimens using plasma exchange, IVIg and/or steroids to achieve disease remission as soon as possible, and then quickly introducing maintenance therapies to reduce requirements for ongoing invasive treatments.

We found a high incidence of other autoimmune diseases (62%) including inflammatory bowel disease (23%), antecedent infections (31%) and surgical instrumentation (15%) in our cohort, as previously reported in other gAChR-positive cohorts from Vernino and Nakane et al.[22, 86] Abdominopelvic instrumentation and inflammation may expose splanchnic autonomic ganglia and induce an aberrant immune response in susceptible individuals. Retrospective studies in GBS have identified surgery in the weeks preceding onset of symptoms in 5-9.5% of patients, with a postulated link between activation of the neuroendocrine stress response, transient immunosuppression, and subsequent infection, with an aberrant immune response against the peripheral nerves due to molecular mimicry.[87, 88]

Following our patients up over several years allowed us to identify occult malignancies in two patients several years after presentation and may have contributed to the higher rate of malignancies observed in our series compared to previous reports (38% v. 17-20%).[22, 86] A deterioration after previously well-controlled disease should prompt repeat screening for possible malignancy.

Five patients were deceased at the time of this study, but we were still able to gather comprehensive retrospective clinical and autonomic data and supplement information by direct correspondence with local physicians. The remaining eight patients were all prospectively evaluated with our full autonomic protocol. We only had longitudinal skin biopsy samples before and after immune therapy for one patient in our cohort but aim to prospectively study more patients with longitudinal samples to assess for changes in autonomic and somatic cutaneous innervation.

As the largest Autonomic Unit in the UK, we were in an ideal position to study this rare disease, but it is possible that some patients with milder disease were solely managed by local teams and not referred to our centre. A referral bias may mean that the patients we studied were on the more severe end of the disease spectrum. There may be patients with milder forms of the disease, that may be more common, and could be amenable to treatment. Whilst our knowledge of the spectrum of autoimmune autonomic failure is still expanding, we would encourage early referral for specialist assessment and multimodal testing, with consideration of treatment on an individual case-by-case basis, with objective longitudinal assessments to quantify treatment response.

In summary, we found objective evidence of severe and widespread autonomic failure in multiple domains in patients with gAChR-positive AAG, with significant improvements after immunotherapy. Quantitative testing with validated autonomic biomarkers should be used to define initial deficits, guide clinical management and monitor treatment response. Early improvements in some domains may reflect potential for more widespread recovery with modifications in immunotherapy. Clinically meaningful functional and pathological recovery is possible even in longstanding disease. The orthostatic intolerance ratio shows promise as a responsive and relevant quantitative biomarker in AAG and may also be useful in other autonomic diseases with emerging treatment options.

Chapter 4. Multimodal biomarkers differentiate gAChR-positive and gAChR-negative autoimmune autonomic failure and PAF

4.1. Introduction

4.1.1 Diagnostic challenges in early presentations of isolated autonomic failure

Patients presenting with isolated autonomic failure in the absence of other neurological deficits represent a diagnostic challenge. 50% of patients with subacute idiopathic autonomic failure have antibodies towards the ganglionic acetylcholine receptor (gAChR),[22] although both patients with and without the antibody can respond to immune therapy.[27] Since the discovery of the gAChR antibody, it has been recognised that a proportion of patients with more gradually progressive disease, previously presumed to have a neurodegenerative disease have detectable gAChR antibodies,[29, 30] and may benefit from immune therapy. Previous studies have shown that patients with the gAChR antibody have widespread severe sympathetic and parasympathetic autonomic failure, with prominent cholinergic deficits.[29, 30] Some gAChR-negative patients presenting with subacute autonomic failure that improve spontaneously or with immune therapy, suggesting an autoimmune aetiology, have been reported to demonstrate sympathetic predominant autonomic failure, more prominent sensory symptoms, and different responses to immune therapy, suggesting a different pathophysiological process.[33, 89]

Patients with insidious onset and gradual progression of autonomic failure without any other neurological and systemic features are given a clinical diagnosis of pure autonomic failure,[14] with a presumed neurodegenerative aetiology, and due to deposition of abnormal alpha-synuclein within the autonomic nervous system, and managed with

symptomatic pharmacological and non-pharmacological therapy, whereas patients with a post-infectious subacute onset of severe autonomic failure are worked up for a possible autoimmune aetiology and treatment with immune therapy. At initial presentation, it may be difficult to differentiate between patients with pure autonomic failure and gAChR-negative autoimmune autonomic disease, due to overlapping clinical features.

In Chapter 3, I used a multi-modal assessment of cardiovascular, neurohormonal, pupillary, bladder, sudomotor and secretomotor autonomic biomarkers with punch skin biopsies to demonstrate patients with gAChR-positive autoimmune autonomic ganglionopathy had a characteristic phenotype of severe sympathetic and parasympathetic autonomic failure, with prominent cholinergic failure and postganglionic denervation, which recovered after immune treatment.[1] For this study, I aimed to use the same multi-modal assessment to compare patients with gAChR-positive and negative patients with autoimmune autonomic failure and patients with pure autonomic failure. My objectives were to:

1. Define the clinical phenotype and objective biomarkers to differentiate between gAChR- positive and gAChR-negative autoimmune autonomic failure and PAF.
2. Explore the pattern of postganglionic somatic and autonomic denervation and recovery following immune therapy in patients with gAChR-positive and gAChR-negative autoimmune autonomic failure

4.2 Methods

4.2.1 Study design

4.2.1.1 Inclusion and exclusion criteria

Patients presenting with autonomic failure and positive gAChR antibodies >100pM were defined as gAChR-positive autoimmune autonomic failure. Patients presenting with a subacute onset of autonomic failure with recovery either spontaneously or following immune therapy with negative gAChR antibodies were defined as gAChR-negative autoimmune autonomic failure, including patients with widespread sympathetic and parasympathetic, sympathetic predominant and parasympathetic cholinergic predominant autonomic failure. Patients with PAF were defined as patients with progressive autonomic failure in the absence of central or peripheral somatic nervous system involvement, other than RBD. Patients with autonomic failure due to diabetes mellitus, chemotherapy, radiotherapy, amyloidosis, other rare genetic diseases and other miscellaneous autonomic disorders e.g., afferent baroreflex disorder, Ross or Harlequin syndrome were excluded.

4.2.1.2 Retrospective study

The clinical notes and investigations for patients presenting to a national autonomic referral centre with autonomic failure between 1987-2021 were reviewed to identify patients with pure autonomic failure and autoimmune autonomic failure, including patients with and without the gAChR antibody. Data was extracted from the clinical history, cardiovascular testing, plasma catecholamines and pupillometry at first evaluation of all patients.

4.2.1.2 Prospective study

From 2018-2021, patients were prospectively recruited to undergo detailed multimodal autonomic testing including cardiovascular autonomic testing, plasma catecholamines, pupillometry, bladder assessment, dynamic sweat testing, tear and saliva testing, and punch skin biopsies. Neurophysiological assessments including nerve conduction studies, EMG, sympathetic skin responses, thermal thresholds and cutaneous silent period were performed as part of routine clinical testing.

All prospectively recruited patients provided written informed consent according to the Declaration of Helsinki. The study design was approved by the London Bridge Research Ethics Committee, REC reference 16/LO/1656).

4.2.2 Cardiovascular autonomic testing and plasma catecholamines

All patients underwent cardiovascular autonomic testing as previously described [55] with beat-to-beat recordings of blood pressure (BP) and heart rate (HR) at rest in the resting supine position and with:

1) Passive head up tilt to 60° for up to 10 minutes
2) Isometric exercise (sustained handgrip for 3 minutes at a third of maximum voluntary contraction pressure)
3) Deep breathing, at a rate of 6 breaths/min
4) Valsalva Manoeuvre (forced expiration at 40mmHg for 10 seconds)

During passive head up tilt, some patients developed severe orthostatic hypotension with signs of cerebral hypoperfusion on orthostasis necessitating early termination. OIR-tilt was calculated by dividing the ΔSBP in mmHg over the time tolerated on head-up tilt in

minutes, up to a maximum of 10 minutes. Valsalva ratio was calculated by dividing maximum heart rate developing during Phase II of the Valsalva manoeuvre over the minimum heart rate occurring within 30 seconds of the peak heart rate.[56] Blood samples were collected via intravenous forearm catheter at rest and following orthostasis for analysis of plasma noradrenaline using high performance liquid chromatography.

4.2.3 Pupillometry

Infrared pupillometry was used to record baseline pupil diameters and responses to stimulation with light impulses and pharmacological stimulation with topical agents including 0.5% apraclonidine, 1% hydroxyamphetamine, 4% cocaine and 0.125% pilocarpine. Absent or diminished pupillary light reflexes and supersensitivity to dilute pilocarpine were indicative of parasympathetic deficits, and delayed pupillary redilation following a light impulse, diminished response to cocaine or supersensitivity to apraclonidine indicated sympathetic deficits. From May 2019, patients were also examined with a prolonged light stimulus to assess for pupillary fatigue, a unique phenomenon previously reported only in patients with gAChR-positive autoimmune autonomic ganglionopathy.[31]

4.2.4 Urinary studies

Urinary flow when voiding with the sensation of a full bladder was assessed by uroflowmetry (Albany Medical SmartFlow) and post-void residual volume measured using a bladder ultrasound scanner (Bardscan Realtime).

4.2.5 Sudomotor and secretomotor testing

Patients underwent DST, an assessment of postganglionic sudomotor function, at the distal leg and forearm bilaterally.[63] After iontophoresis with 1% pilocarpine, skin was

coated with iodine and formation of sweat gland imprints on starch covered tape was recorded. Density of activated sweat glands/cm^3, sweat output/min/cm^3, and average sweat output/gland was calculated for each site and the mean value for both sides used. A length dependent deficit was defined as sweat output at the distal leg site less than the 5^{th} percentile of normal values and less than 1/3 of sweat output at the forearm.[90] Lacrimal production was measured using Schirmer's test and average for both eyes calculated.[64] Salivary production was measured using the unstimulated salivary production test.[65]

4.2.6 Morphological analysis of punch skin biopsies

Patients had 3-mm punch skin biopsies collected from the distal leg and anterior thigh. To maximise the sampling of arrector pili muscles and sweat glands, biopsies were centred around a hair follicle. Samples were placed into chilled Zamboni solution and then transferred to cryoprotectant solution after 4-6 hours. Samples were sliced into 50µm thick sections using a freezing sliding microtome (Leica 2000) and processed for indirect immunofluorescence according to standard procedures using a large panel of antibodies, including primary antibodies against collagen type IV (ColIV), protein gene product (PGP) 9.5, a pan-neuronal marker, vasoactive peptide (VIP), a cholinergic marker, dopamine beta hydroxylase (DBH), an adrenergic marker, and phosphorylated synuclein (p-syn), and species-specific secondary antibodies coupled with Cy2, Cy3 and Cy5 fluorophores.[91] Digital confocal images were acquired using a non-laser confocal system (Apotome2 Zeiss, Jena, Germany, EU).

4.2.7 Quantification of intraepidermal and pilomotor fibres

IENF density was measured as the number of fibres crossing the dermal-epidermal junction according to current guidelines.[69] Quantification of pilomotor fibres was performed as previously described.[39] Briefly, arrector pili muscle segments parallel to the focal plane were acquired using a 20x objective. The single optical section with the most fibres running for at least 100 µm parallel to the major axis of the muscle was selected from the Z-stack. A line was then traced perpendicular to the major axis intercepting the most fibres. PNF density was calculated as the average number of intercepts per muscle width in fibres/mm of all muscles suitable for quantification for each staining for each biopsy.

4.2.8 Statistical analysis

Data were captured electronically using a secure Research Electronic Data Capture (REDCap) platform. Statistical analysis was performed using R Studio, Version 1.2.1335. Summary data have been displayed as median, interquartile range for continuous data and numbers, percentages for categorical data. Distributions of data were assessed for normality by visual inspection and using Shapiro-Wilk tests. Pairwise comparisons were made with unpaired two-tailed T-tests/Wilcoxon rank-sum tests and group comparisons were made with ANOVA/Kruskal-Wallis tests with post-hoc comparisons using Tukey/Dunn's tests with Bonferroni corrections as appropriate. Chi-squared tests were used to compare categorical data. Spearman's rank correlation was used to assess the correlation between linear variables. $P<.05$ was considered significant.

4.3 Results

4.3.1 Patient recruitment

200 patients were included in the retrospective study: 54 with autoimmune autonomic failure, 20 that were gAChR-Ab-positive, and 34 that were gAChR-negative, and 146 with PAF. Cardiovascular testing was performed in all 200 patients in the retrospective study, plasma catecholamines in 187 patients, and pupillometry in 98 patients. 55 patients were prospectively studied with additional bladder studies, secretomotor and sudomotor testing, and skin biopsies: 27 patients with autoimmune autonomic failure, 14 that were gAChR-positive and 13 that were gAChR-negative, and 28 patients with PAF.

Amongst the retrospective cohort, 98 patients (49%) were female, with no difference in sex distribution between groups. Patients with autoimmune autonomic failure were significantly younger at first assessment (median age 55-56 years) compared to patients with PAF (median age 68, $P<.0001$) (Table 4.1).

4.3.2 Clinical features

In the prospectively recruited group, antecedent infections or surgical instrumentation were common in the patients with autoimmune autonomic failure, both gAChR-positive (57%) and gAChR-negative groups (62%). Other autoimmune diseases were reported in 53% of gAChR-negative and 36% of gAChR-positive autoimmune autonomic failure groups, in contrast to only 7% of the PAF group ($P<.05$). Malignancies were found in of one the gAChR-positive (7%), two of the gAChR-negative (15%) and four of the PAF patients (14%). The malignancies were found during the work-up of the autoimmune patients, whereas in the PAF group they were detected and treated either prior to their illness, or several (7-30) years after onset of symptoms. 57% of patients with PAF

reported symptoms consistent with RBD, and 21% reported hyposmia/anosmia, which were uncommon or not present in the autoimmune groups (Table 4.1).

4.3.3 Cardiovascular testing and plasma noradrenaline

As previously described, gAChR-positive patients had a consistent autonomic phenotype with severe sympathetic and parasympathetic autonomic failure on cardiovascular autonomic testing. In comparison, the gAChR-negative patients had less severe fall in systolic blood pressure on tilt (median ΔSBP 58 mmHg [IQR 33-68 mmHg] vs 76 mmHg [52-99 mmHg], *P*=.01), and greater compensatory heart rate rise with tilt (15bpm [6-30bpm] vs 3 bpm [0-11 bpm], *P*=.001) and with isometric exercise (7bpm [0-11bpm] vs 3 [0-3bpm], *P*=.001). gAChR-negative patients also had greater heart rate changes with deep breathing (6 bpm [3-9bpm] vs 3 [0-4], *P*=.01) and Valsalva ratio (1.33 [1.13-1.41) vs 1.04 [1.02-1.10], *P*=.0001), reflecting more preserved parasympathetic cardiovagal function (Table 4.2).

When compared to the gAChR-negative patients, PAF patients had a higher supine blood pressure (152mmHg [136-173 mmHg] vs 138mmHg [121-151mmHg], *P*=.01), ΔSBP (71mmHg [55-90 mmHg] *P*=.001, and lower HR_{DB} (3bpm [0-7bpm], *P*=.01), and lower supine noradrenaline 176 pg/ml [142-204pg/ml] vs 190 pg/ml [172-214pg/ml], *P*=.03).

Table 4.2. Comparison of clinical features in prospectively recruited gAChR-positive, gAChR-negative AAG and PAF patients

	Autoimmune autonomic failure		
	gAChR-positive	gAChR-negative	PAF
n	14	13	28
Female, n, %	6, 43%	8, 61%	9, 32%
Age, y, at recruitment	51, 41-60	47, 31-58	61, 55-70
Antecedent event	8, 57% - Resp. infections (4) - Gastroenteritis - UTI - Colectomy - Urethral dilation	8, 62% - Gastroenteritis (3) - Flu-like (2) - Colectomy - Endoscopy	
Other antibodies	2, 14% - Anti-Hu - Anti Voltage-gated calcium channel	4, 31% - Anti-Ro/La, RhF, ANA 1:1280 - Anti-Ro, anti-Purkunje - Anti-Ro - Anti-GFAP (CSF)	
Autoimmune disease	5, 36% - Hypothyroidism (2) - Crohn's, Addison's hypothyroidism, alopecia - UC, psoriasis, Addison's hypothyroidism, - UC	7, 53% - Sjogren's (3) - UC, uveitis - Hypothyroidism - Alopecia - Polymyalgia rheumatica	2, 7%[1] - Hypothyroidism - Inflammatory arthritis
Malignancy	1, 7% - Ovarian teratoma, 5y after onset	2, 15% - Parotid MALToma, <1y after onset - Thyroid ca, 2y after onset	1, 3.6% - Colorectal ca, before onset 3, 8%, several years after onset of symptoms - Breast DCIS, 7y after onset - Multiple myeloma, lung adenoca, 13y/15y after onset - Breast ca, 30y after onset
RBD*	0, 0%	1, 8%	16, 57%[2]
Hyposmia*	0, 0%	0, 0%	6, 21%

*UTI, urinary tract infection; UC, Ulcerative colitis; ANA, anti-nuclear antibody; MALToma, mucosa-associated lymphoid tissue lymphoma; [1]significantly less than gAChR-negative (P=.003) and gAChR-positive groups (P=.02), [2]significantly more than gAChR-negative group (P=.003). *based on clinical history, not formally assessed.*

Table 4.3. Demographic details, cardiovascular testing, plasma noradrenaline, and pupillometry in gAChR-positive, gAChR-negative AAG and PAF patients in retrospective study

	Median, IQR			*P*-value			
	Autoimmune autonomic failure (54)						
	gAb+ (20)	gAb- (34)	PAF (146)	ANOVA	gAb+ vs gAb-	gAb+ vs PAF	PAF vs gAb-
Age, years	55, 41-63	56, 44-66	68, 59-75	<.0001	1	<.0001	<.0001
Female n, %	8,40%	19, 55%	71, 49%	.52			
Supine							
SBP, mmHg	150, 139-160	138, 121-151	152, 136-173	.01	.59	.52	.01
HR, bpm	65, 63-67	70, 65-75	67, 60-73	.09			
Head-up tilt							
Δ SBP, mmHg	76, 52-99	58, 33-68	71, 55-90	.001	.01	.76	.001
Δ HR, bpm	3, 0-11	15, 6-30	9, 3-17	.002	.001	.07	.045
Isometric exercise							
Δ SBP, mmHg	0, -4 to 6	5, -5 to 18	3, -4 to 9	.48			
Δ HR, bpm	1, -1 to 3	7, 0-11	3, 1-7	.002	.001	.02	.23
HR_{DB}, bpm	3, 0-4	6, 3-9	3, 0-7	.004	.01	.74	.01
Valsalva ratio	1.04, 1.02- 1.10	1.33, 1.13-1.41	1.15, 1.07-1.32	<.0001	<.0001	.002	.10
Noradrenaline, n	20	32	135				
Supine, pg/ml	176, 159-190	190, 172-214	176, 142-204	.03	.29	1	.03
Δ NA, pg/ml	10, 3-18	14, 7-33	10, 2-21	.16			
Pupils, n	19	21	58				
Normal	0, 0 %	4, 19%	17, 29%	.01	.03	.01	
Sympathetic	2, 11%	9, 43%	37, 64%	.0001	.03	<.0001	
Parasymp.	0, 0%	1, 5%	4, 7%	.82			
Both symp. + parasymp	17, 89%	7, 33%	0, 0%	<.0001	.0004	<.0001	<.0001

gAb+/-, ganglionic acetylcholine receptor antibody positive/negative; PAF, pure autonomic failure; SBP, systolic blood pressure; HR, heart rate; NA, noradrenaline; Symp., sympathetic; Parasymp., parasympathetic

4.3.4 Pupillometry

All gAChR-positive patients had abnormal pupils, with the majority (89%) having both sympathetic and parasympathetic pupillary deficits and a minority having isolated sympathetic deficits (11%). In contrast, only 33% of gAChR-negative patients and 0% of PAF patients had both sympathetic and parasympathetic pupillary deficits (*P*<.0001), and 43% of gAChR-negative, patients and 64% had sympathetic pupillary deficits only (*P*<.0001), and 19% of gAChR-negative patients and 29% of PAF patients had normal pupils (*P*=.008).

Amongst the patients tested for pupil fatigue from May 2019, all 12 gAChR-positive patients had this finding, which was not present in any gAChR-Ab-negative (n=7) or PAF (n=17) patients.

4.3.5 Bladder studies

Over 70% of the autoimmune patients had elevated post-void residual volumes >100ml (71% of gAChR-positive and 75% of gAChR-negative patients), compared to only 38% of the PAF group (*P*<.05), with 50% of gAChR-positive and 46% of gAChR-negative patients requiring catheters, compared to 25% of PAF patients (Table 4.3).

). Where patients were able to void spontaneously and generate a uroflowmetry profile, this was abnormal in 92% of the gAChR-patients, 63% of the gAChR-negative patients, and 65% of the PAF patients. The autoimmune patients with abnormal uroflow typically had intermittent or polyphasic uroflow patterns (10/11 gAChR-positive, 4/5 gAChR-negative) in keeping with neurogenic voiding difficulties, whereas in the PAF group this was more mixed, with 6/13 having intermittent/polyphasic voiding patterns and 7/13 with prolonged/flattened profiles, which can also be seen with obstructive outflow problems (Figure 4.1). Of note, the gAChR-positive patient and 2 of the 3 gAChR-negative patients with normal uroflow profiles had these performed after spontaneous remission or on maintenance immunotherapy.

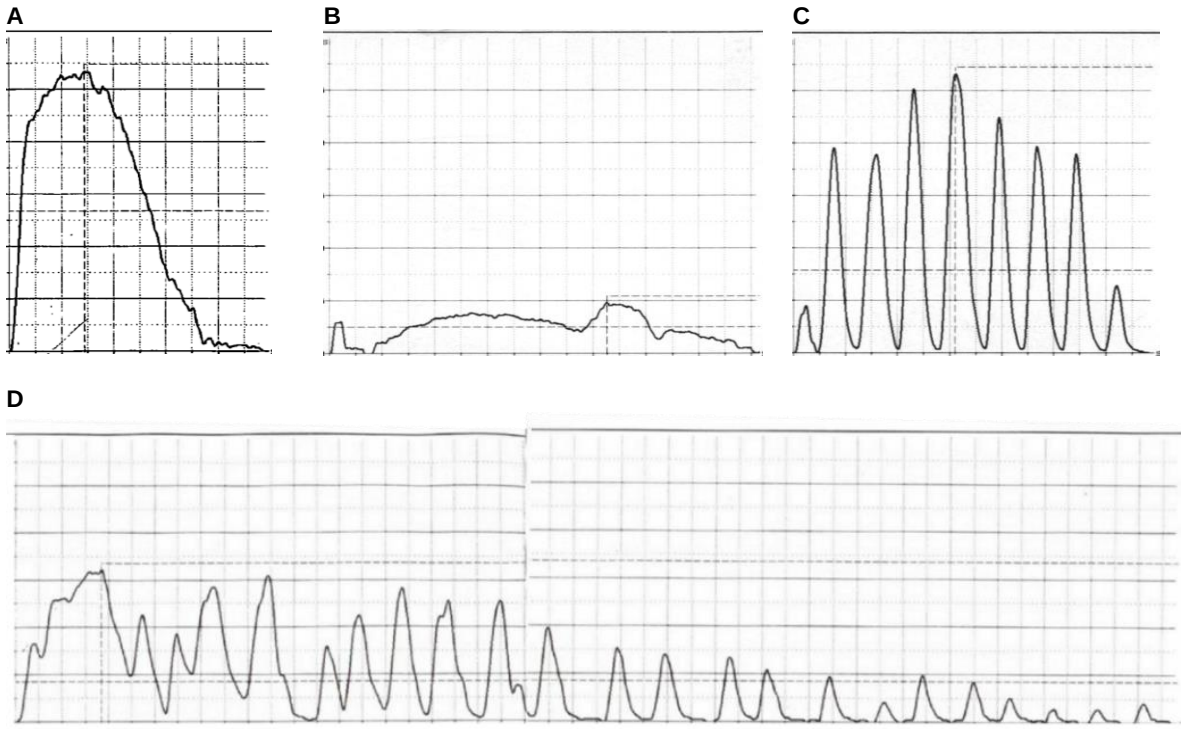


Figure 4.5. Examples of uroflow profiles in the patient cohort. A) normal, bell-shaped profile. B) flattened/prolonged profile, typically seen in obstructive urinary outflow problems. C) Polyphasic D) Polyphasic and intermittent uroflow profiles, suggesting abdominal straining to try overcome poor detrusor contractility.

4.3.6 Secretomotor testing

Most gAChR-positive patients (86%) had abnormal saliva production <0.1g/min, a significantly greater proportion than in the gAChR-negative (35%, *P*=.01) and PAF patients (37%, *P*=.02). Reduced mean tear production ≤ 5mm on Schirmer's testing was commonly found in all groups (64%, 72% and 56% of gAChR-positive, gAChR-negative and PAF groups respectively).

4.3.7 Dynamic sweat testing

Most patients had abnormal post-ganglionic sudomotor function on DST. Mean sweat output was reduced in all PAF and gAChR-negative patients, and 92% of gAChR-positive patients at the distal leg. At the forearm, mean sweat output was reduced in 90% of PAF patients, 86% of gAChR-positive and 73% of gAChR-negative patients. A non-length

dependent deficit was more commonly seen in the gAChR-patients (79%) in comparison to the gAChR-negative (45%) and PAF patients (47%), although this was not significant at the 5% level (*P*=.09).

4.3.8 NCS/EMG

None of the gAChR-positive or PAF patients a generalised large fibre neuropathy on NCS/EMG. Three gAChR-negative patients had abnormal NCS/EMG, with different clinical phenotypes summarised in Box 4.1. The first had post-infectious sensory and autonomic symptoms with severe widespread autonomic failure and a length-dependent sensory>motor axonal neuropathy. The second had meningoencephalitis, severe sympathetic predominant autonomic failure, and a sensory neuronopathy with positive CSF Glial fibrillary acidic protein (GFAP) antibodies. The third had a non-length dependent sensory neuronopathy, autonomic failure, with positive Schirmer testing and anti-Ro antibodies in keeping with Sjogren's disease.

Table 4.4. Bladder, secretomotor, dynamic sweat testing and neurophysiology

	Autoimmune autonomic failure, n=27		PAF n=28
	gAChR-positive, n=14	gAChR-negative, n=13	
Catheter use	7, 50%	6, 46%	7, 25%
Bladder testing, n	14	12	24
Post-void residual volume >100ml	10, 71%	9, 75%	9, 38%[1]
Uroflow pattern			
Unable to void, catheter dependent	2, 14%	4, 31%	4, 17%
Normal	1[a], 8%	3[b], 38%	7, 35%
Abnormal	11, 92%	5, 63%	13, 65%
Intermittent / polyphasic	10	4	6
Prolonged / flattened	1	1	7
Secretomotor testing, n	14	11	18
Tear production, mm	3.5, 0-11.4	1.3, 0.0 -1.2	4.8, 2.9-9.9
≤5mm	9, 64%	8, 72%	10, 56%
Saliva production, g/min	0.01, 0.00-0.02	0.17, 0.01-0.26	0.13, 0.04-0.20
<0.1g/min	12, 86%[2]	4, 35%	7, 37%
Dynamic Sweat testing, n	14	11	26
Forearm, n	14	11	19
Sweat output, N ≥ 624 nL/cm^2/min	196, 70-522	354, 62-869	160, 98-434
Sweat glands/cm^2, N ≥ 100 glands/cm^2	52, 29-99	94, 21-124	64, 58-114
Sweat output/gland, N ≥ 5.6 nL/min	4.2, 1.8-6.1	3.7, 2.1-6.8	2.6, 1.5-5.2
Distal Leg, n	11	11	26
Sweat output, N ≥ 417 nL/cm^2/min)	85, 51-300	123, 13-285	75, 43-147
Sweat glands/cm^2, N≥ 64 glands/cm^2	40, 28-53	39, 21-73	38, 27-55
Sweat output/gland, N ≥ 5.6 nL/min)	2.2, 1.7-5.4	3.7, 0.7-4.6	2.1, 1.1-3.4
Length dependent deficit[c]	3, 21%	6, 55%	10, 53%
Neurophysiology	14	10	26
NCS/EMG abnormal	0, 0%	3, 30% [3]	0, 0%
Sympathetic skin responses absent/reduced	6/14, 43%	5/9, 56%	17/25, 68%
Cutaneous silent period abnormal	3/10, 30%	2/8, 25%	5/24, 20%
Thermal thresholds elevated	2/12, 17%	2/9, 22%	8/24, 33%

[a] On maintenance immune therapy; [b] 2 patients after spontaneous remission; [c] Sweat output at leg <1/3 of arm. [1]significantly less than gAChR-positive group (P=.04) and gAChR-negative group (P=.03),[2]significantly more than gAChR-negative (P=.01) and PAF group (P=.02), [3]significantly more than PAF group (P=.02).

Box 4.1. gAChR-negative patients with abnormal NCS/EMG

1. A 65-year-old man presented after a gastrointestinal illness with multiple collapses, constipation, weight loss, hypohidrosis, urinary retention, erectile dysfunction, painful paraesthesia, with loss of distal pinprick, vibration and lower limb deep tendon reflexes and preserved muscle strength. He had widespread cardiovascular autonomic failure, parasympathetic pupillary deficits, reduced supine noradrenaline and tear production (B1, Table 4.7), elevated CSF protein 1.19g/L, and a length-dependent sensory>motor axonal neuropathy, confirmed on sural nerve biopsy, with no overt features of vasculitis. After 2g/kg IVIg, he improved over four months, voiding spontaneously, with no orthostatic hypotension, normal supine noradrenaline and rise with tilt after one year.

2. A 17-year-old man presented with meningoencephalitis, severe orthostatic intolerance, constipation, and urinary retention, with sympathetic predominant autonomic failure with severe neurogenic OH (ΔSBP 50mmHg within 2 minutes of tilt) with relatively preserved HR_{DB} (16bpm) and supine noradrenaline (204pg/ml) with no rise on tilt, bilateral Horner's syndrome and reduced tear production. Lower limb reflexes and sensory action potentials were absent, with normal motor studies. MRI brain showed T2 hyperintense lesions in the splenium. CSF showed lymphocytic pleocytosis with positive GFAP antibodies. After intravenous then oral steroids he made a good recovery with no OH on repeat testing five months after treatment.

3. A 62-year-old man presented with distal paraesthesia and numbness, sicca syndrome, pre-syncopal symptoms and urinary retention. Investigations showed positive Schirmer's test, anti-Ro antibodies, non-length dependent sensory neuropathy with normal motor studies, with neurogenic orthostatic hypotension on standing and reduced heart rate variability with deep breathing, bilateral Horner's syndrome, consistent with a Sjogren's related sensory and autonomic neuropathy,[92] with improvements on oral hydroxychloroquine treatment on repeat testing 2 years after initial assessment.

4.3.9 Summary of differences in clinical phenotype between groups

In summary, as expected, patients with autoimmune autonomic failure, both gAChR-positive and negative, presented at a younger age compared to patients with PAF, and frequently reported antecedent events, other autoimmune diseases, urinary retention, and intermittent uroflowmetry profiles in keeping with neurogenic voiding dysfunction. In contrast, patients with PAF were older at first assessment, more commonly reported symptoms consistent with RBD, in keeping with a neurodegenerative alpha-synucleinopathy, and less frequently had urinary retention, with a mixture of normal, low volume, prolonged, intermittent and polyphasic uroflowmetry profiles, suggesting a combination of obstructive and neurogenic voiding dysfunction, as expected in an older cohort.

gAChR-positive patients consistently had severe widespread sympathetic and parasympathetic cardiovascular autonomic failure, with prominent cholinergic deficits over cardiovascular, pupillary, urinary, secretomotor testing, with pupil fatigue and non-length dependent post-ganglionic sudomotor dysfunction, in keeping with pathology involving sympathetic and parasympathetic ganglia. In contrast, gAChR-Ab-positive patients had a more variable clinical phenotype, but more commonly demonstrated sympathetic predominant autonomic failure, with relatively preserved HR_{DB}, Valsalva ratio, pupillary parasympathetic function, and saliva production compared to gAChR-Ab positive patients, with just under a third having evidence for a peripheral large fibre neuropathy or sensory neuronopathy.

Table 4.4. Summary of differences in clinical phenotype between groups

Clinical phenotype	gAChR-positive	gAChR-negative	PAF
Age <60 years at first presentation	+++	+++	+
Antecedent events	+++	+++	-
Other autoimmune disease	++	+++	+
RBD	-	+	+++
HR_{DB} <10bpm	+++++	++++	+++++
Valsalva ratio < 1.2	+++++	++	+++
Pupils			
Sympathetic + parasympathetic deficits	+++++	++	-
Sympathetic	+	+++	++++
Normal	-	+	++
Pupil fatigue	+++++	-	-
Urinary retention >100ml	++++	++++	++
Catheter use	+++	+++	++
Reduced saliva production <0.1g/min	+++++	++	++
Length-dependent DST abnormality	++	+++	+++
Abnormal NCS/EMG	-	++	-

+, 1-20%;
++, 21-40%;
+++, 41-60%;
++++, 61-80%;
+++++, 81-100%.

4.3.10 Cutaneous somatic and autonomic innervation in gAChR-positive and gAChR-negative autoimmune autonomic failure

Given the differences in clinical phenotype between gAChR-positive and gAChR-positive patients, we postulated that there would also be differences in the postganglionic somatic and autonomic innervation evaluated through punch skin biopsies. 22 of our prospectively studied patients had skin biopsies collected from the distal leg and anterior thigh (14 gAChR-positive and 8 gAChR-negative). Age, sex distribution and disease

duration at the time of baseline skin biopsies did not differ significantly between groups (Table 4.5Table 4.5).

4.3.10.1 IENF density

In baseline samples collected at recruitment, we commonly observed a severe loss of cutaneous intra-epidermal innervation, with about half of the patients in both groups showing a length dependent reduction in IENF (46% gAChR-positive; 50% gAChR-negative groups, Table 4.5).[34] Only one gAChR-positive patient (patient 4) had preserved IENF density within 5% cut-of values for age and sex at the leg,[80] and three gAChR-positive (patients 2, 3, and 4) and one gAChR-negative patient (B7) had preserved IENF at the thigh.[34] The three gAChR-positive patients had a relatively short disease duration (2.8-3.8 years), and the gAChR-negative patient was on maintenance immune therapy at the time of biopsy (Table 4.6).

4.3.10.2 Morphological changes

Of note, even in samples with relatively normal IENF density, the fibres were unevenly spatially distributed, with stretches of denervated epidermis and clusters of branching fibres sprouting from some subepidermal plexuses, suggesting regenerative attempts.[93] The fibres had a markedly irregular, beaded appearance, as previously described.[1] This appearance was frequently seen in gAChR-positive patients, but also in one of the gAChR-negative patients (B7) with a similar phenotype to gAChR-positive group, with pandysautonomia without any central or peripheral somatic deficits, with sympathetic and parasympathetic cardiovascular autonomic failure, sympathetic pupillary deficits, urinary retention, and reduced tear and saliva production (Table 4.6).

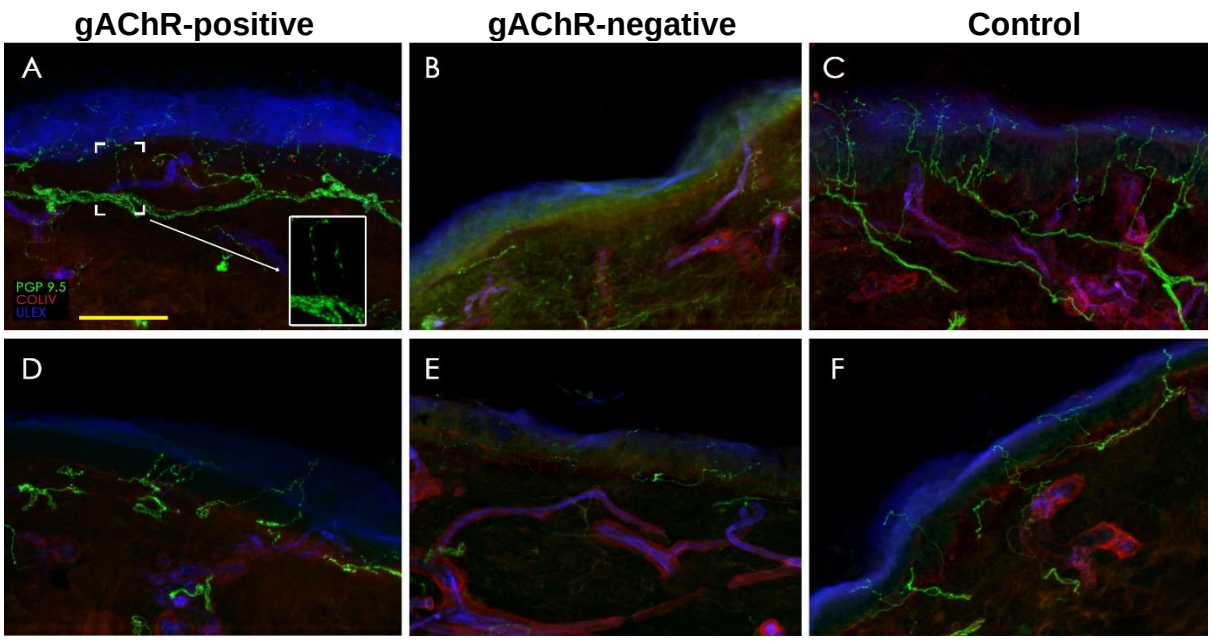


Figure 4.2. Skin biopsies from the thigh (A, B, C) and leg from a gAChR-positive patient (Patient 4), a gAChR-negative patient (Patient B2), and healthy control. Even in patients with relatively preserved IENF density, we commonly observed a very abnormal beaded and fragmented appearance in the cutaneous nerves (A, D, inset box in A shows higher magnification). This striking pattern was frequently observed in the gAChR-positive patients, but also in one of the gAChR-negative patients with pandysautonomia. Amongst both gAChR-positive and gAChR-negative groups, some patients had a severe loss of sub-epidermal plexuses and intra-epidermal nerve fibres as shown in Parts B and E. For comparison, Parts C and F show the regular and even staining of sub-epidermal plexuses and intra-epidermal nerves in the thigh and distal leg of a healthy control.

4.3.10.3 Pilomotor innervation

Both groups also demonstrated a marked reduction in the innervation of arrector pili muscles. At the distal leg, pilomotor nerve fibre (PNF) density was reduced in all patients below normal values with PGP, VIP, and DBH staining.[39] At the thigh, PNF density with pan-neuronal marker PGP was relatively preserved in two gAChR-patients with a short disease duration (patient 3, 4) and within normal cut-off values in patient 14, who was on immune therapy. However, PNF density with cholinergic and adrenergic specific markers VIP and DBH in all patients.

Some of the gAChR-positive patients with shorter disease duration (patients 2, 3) had almost complete loss of VIP-ir cholinergic pilomotor fibres, with relatively preserved DβH-

ir adrenergic pilomotor innervation, suggesting a preferential early loss of cholinergic fibres (Figure 4.3). In some of the gAChR-negative patients, with sympathetic predominant autonomic failure, the opposite pattern was seen, with almost complete loss of DβH-ir adrenergic fibres (Patients B4, B5) and relative preservation of VIP-ir cholinergic fibres, suggesting a preferential loss of post-ganglionic adrenergic fibres (Table 4.6, Table 4.7). Overall, PNF density with DβH was significantly lower in gAChR-negative patients compared to gAChR-positive patients at both the distal leg (median 0 [IQR 0-0] f/mm vs 4.6 [IQR 0.1-14.7] f/mm, *P*=.03) and thigh (0 [IQR 0-7.6] f/mm vs 16.1 [IQR 3.7-19.9] f/mm *P*=.05) (Table 4.5)

Table 4.5. Intraepidermal and pilomotor innervation at recruitment in gAChR-positive and gAChR-negative patients

	Median, IQR		*P*-value
	gAChR-positive	gAChR-negative	
n	14	8	
Female, n, %	6, 43%	5, 63%	.47
Age at onset, y	44.8, 31.4-51.5	27.0, 21.5-43.4	.20
Age at first biopsy, y	51.4, 40.7-60.4	51.9, 36.0-59.1	.56
Disease duration, y	4.7, 3.5-9.3	13.0, 1.1-27.0	.60
IENF density, f/mm			
Distal leg	1.2, 0.4-3.1[1]	0.7, 0.2-2.5	.48
Thigh	1.6, 0.9-2.3[3]	1.3, 0.4-2.4[1]	.49
Length dependent loss,* n, %	6/13, 46%	4/8, 50%	.65
Pilomotor fibre density, f/mm			
Distal leg			
PGP (N>67.2)	25.9, 18.7-31.3	18.4, 0.0-26.3	.45
VIP (N>44.4)	4.4, 0.0-12.3	0.0, 0.0-18.2	.78
DβH (N>38.7)	4.6, 0.1-14.7	0.0, 0.0-0.0	.03
Thigh			
PGP (N>60.7)	30.7, 27.5-37.5[1]	22.0, 9.4-33.2	.14
VIP (N>35.1)	4.3, 2.3-2.1	0.9, 0.0-13.9	.49
DβH (N>34.5)	16.1, 3.7-19.9	0.0, 0.0-7.6	.02

*[1-3] Numbers of patients with innervation within normal cut-off values for age and sex; * IENF at leg/thigh <0.48[34]*

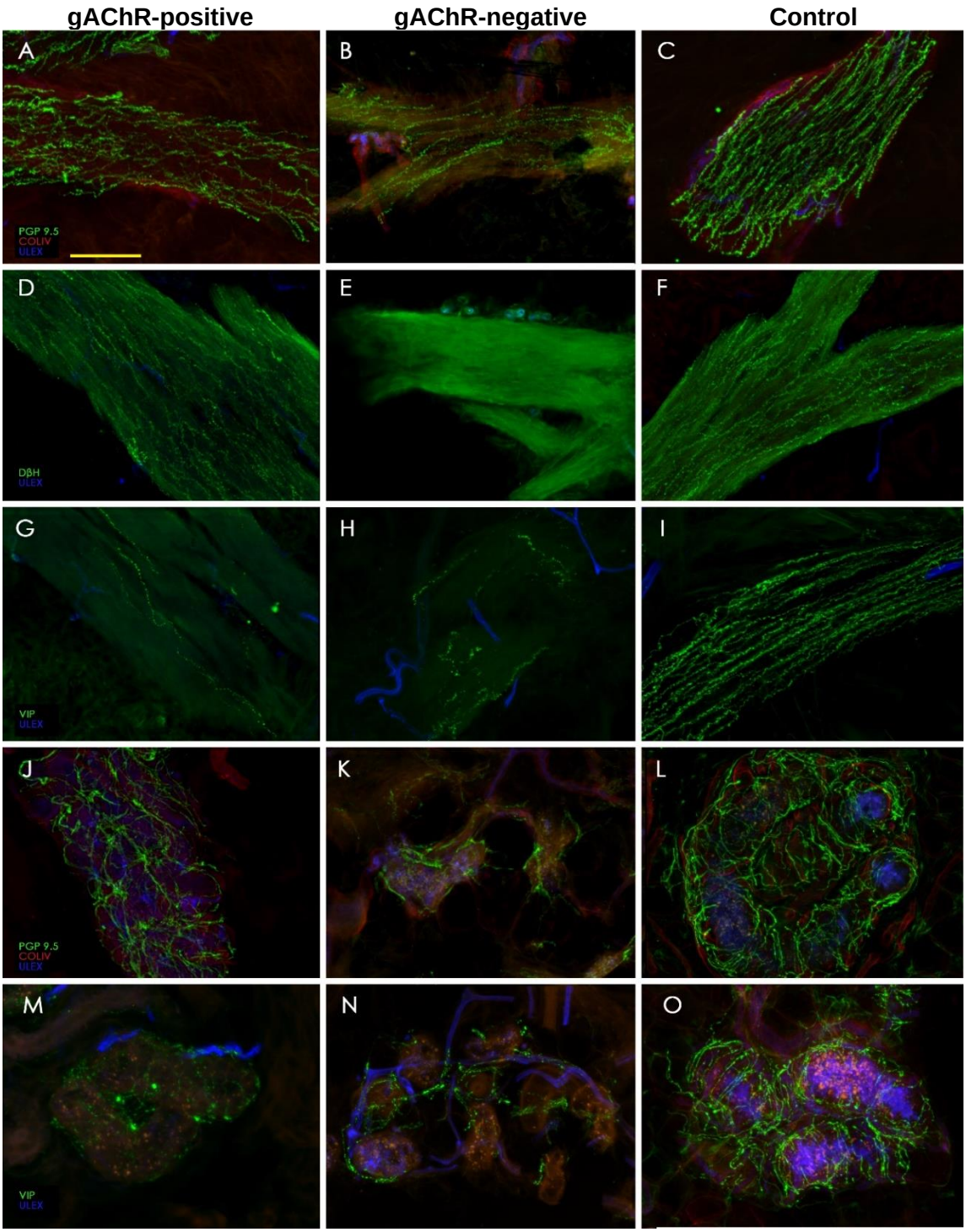


Figure 4.3. Autonomic innervation in gAChR-positive (Patient 3; A, D, G, J, M) and gAChR-negative patients (Patient B5; B, E, H, K, N,) and healthy control (C, F, I, L, O). Samples from patient 3 were taken <3y after onset and demonstrated relatively preserved innervation of arrector pili muscles and sweat glands with PGP (A, J), but a severe loss of VIP-ir pilomotor and sudomotor fibres (G, M). DβH-ir pilomotor fibres were relatively preserved (D). Biopsies from patient B5 were taken 15y after onset of severe sympathetic autonomic failure with gradual spontaneous recovery. Again, there is relative preservation of PGP-ir pilomotor fibres (B), but no DβH-ir adrenergic fibres (E), and few VIP-ir cholinergic fibres (H). The sweat glands have an abnormal morphology with loosely coiled tubules with few PGP-ir and VIP-ir fibres (K, N). The samples from a healthy control show an abundance of PGP-ir, DβH-ir, and VIP-ir pilomotor fibres, and tightly coiled sweat tubules, richly innervated with PGP-ir and VIP-ir sudomotor fibres.

Table 4.6. Demographic details, immune treatment, and intraepidermal and pilomotor innervation in individual patients at recruitment

				Immuno therapy at biopsy	Leg				Thigh				IENF leg/ thigh
					IENF ,f/mm	Mean PNF, f/mm			IENF , f/mm	Mean PNF, f/mm			
	Sex	Age , y	Disease duration, y			PGP	VIP	DBH		PGP	VIP	DBH	
	gAChR-positive, n=14												
1	F	63	0.9	PLEX, IVMP, Pred	0.2	11.8	0.0	0.0	0.7	19.9	12.7	1.3	0.31[l]
2	M	62	2.8		6.1	36.7	0.0	14.4	19.2[n]	34.8	12.8	25.2	0.32[l]
3	M	35	2.8		9.2	40.3	0.0	19.2	17.8[n]	56.9	3.4	29.2	0.52
4	M	51	3.8		10.8[n]	35.3	19.2	16.0	30.7[n]	48.6	8.7	25.5	0.35[l]
5	M	57	4.0		0.8	21.2	8.7	0.0	1.7	31.6	1.3	19.2	0.49
6[r]	F	51	4.3		1.5	25.9	20.1	15.5	0.7	28.7	12.1	16.1	2.16
7	M	52	4.6		0.5	27.8	12.3	1.2	0.5	26.3	0.0	0.0	0.91
8[r]	F	39	4.8		0.2	22.0	0.0	5.7	1.0	37.5	3.4	15.4	0.18[l]
9[r]	F	67	6.8		3.6	16.6	2.5	NA	1.6	15.0	4.3	6.5	2.18
10	M	39	7.9	IVIg, PLEX, MTX	0.6	14.3	0.0	0.5	1.6	28.9	0.0	0.0	0.35[l]
11	F	41	9.8		1.5	20.9	0.0	NA	1.5	30.7	7.4	6.1	0.96
12	M	61	18.5		0.6	15.7	6.3	3.5	NA	NA	NA	NA	NA
13	M	51	25.9	SR, HCT	0.3	29.6	12.3	6.5	1.1	29.0	3.3	18.7	0.26[l]
14[r]	F	55	33.7	PLEX	1.9	31.3	25.0	0.0	2.3	73.2[n]	16.1	19.9	0.82
n, % within normal range					1, 7	0, 0	0, 0	0, 0	3, 21	1, 7	0, 0	0, 0	6[l], 46
	gAChR-negative, n=8												
B1	M	65	0.2	Pred, IVIg	0.0	0.0	0.0	0.0	0.3	9.3	0.0	0.0	0.00[l]
B2[r]	M	42	0.3	PLEX	1.5	0.0	0.0	0.0	1.6	9.6	0.0	0.0	0.89
B3	F	23	3.7	PLEX	0.7	26.3	18.9	0.0	0.9	34.5	1.5	10.0	0.73
B4	F	34	10.7	AZA	0.2	17.2	14.4	0.0	0.7	24.6	25.0	0.0	0.22[l]
B5	F	59	15.4	SR	2.8	40.5	18.2	0.0	2.6	29.1	15.0	0.0	1.07
B6	F	47	25.5		0.6	NA	NA	NA	0.2	0.0	0.0	0.0	4.00
B7	F	58	27.5	AZA, HCQ	7.4	25.6	0.0	19.1	17.7[n]	59.5	10.6	21.1	0.42[l]
B8	M	57	35.2		0.5	18.4	0.0	0.0	1.7	19.3	0.2	0.0	0.31[l]
n, % within normal range					0, 0	0, 0	0, 0	0, 0	1, 12	0, 0	0, 0	0, 0	4[l], 50

PLEX, plasma exchange; Pred, prednisolone; MTX, methotrexate; SR, HCT, spontaneous recovery, further stabilisation with hydrocortisone for Addison's disease; AZA, azathioprine; HCQ, hydroxychloroquine; NA – no pilomotor muscles suitable for quantification, [l]length dependent deficit, IENF at leg/thigh <0.48,[34] [r]repeat samples available, [n]within 5% normal cut off for age/sex,[34, 39, 80].

Table 4.7. Multi-modal autonomic testing for gAChR-positive and gAChR-negative patients at time of biopsies

			Head-up tilt										
Pt	Sex	Age, y	Δ SBP	TT, min	HR_{DB}	VR	Sup. NA	ΔNA	Pupil	Tear	Saliva	Catheter/ PVR	Comment
1	F	63	90	10	4	1.01	163	13	Both	0.0	0.11[n]	304	
2	M	62	76	2	0	1.05	184	3	Both	26.5	0.00	C	
3	M	35	54	2	0	1.09	189	11	Both	10.0	0.01	237	
4	M	51	65	10	0	1.02	163	2	Both	0.0	0.00	623	
5	M	57	32	10	3	1.00	163	1	Both	24.0	0.00	588	
6[r]	F	51	26	5	7	0.92	166	14	Both	1.0	0.00	169	
7	M	52	68	3	0	1.11	178	3	Both	15.5	0.04	327	
8[r]	F	39	73	10	5	1.32	190	13	Both	4.5	0.01	140	
9[r]	F	67	33	1	0	1.03	135	2	Both	0.0	0.01	73[n]	
10	M	39	62	10	5	1.18	362*[n]	20*	Both	10.0	0.02	0[n]	
11	F	41	84	3	5	0.95	137	1	Both	5.0	0.00	320	
12	M	61	45	10	4	1.36	277*[n]	14*	Symp	2.5	0.00	C	
13	M	51	6[n]	10	5	1.62[n]	204[n]	117[n]	Both	0.0	0.19[n]	76[n]	SR, HCT
14[r]	F	55	46	2	0	1.05	188	13	Both	0.0	0.02	0[n]	
n, % normal			1, 7		0, 0	1, 7	3, 21	1,7	0, 0	5, 36	2,14	4, 29	
B1	M	65	64	2	3	1.09	162	5	Para-symp	0.5	0.20[n]	C	S>M neuropathy
B2[r]	M	42	58	10	26[n]	1.35	163	3	Symp	2.5	0.26[n]	0	Sympathetic
B3	F	23	-10[n]	10	22[n]	2.18[n]	253[n]	218[n]	Norm	19.0	0.17[n]	C	Bladder/SM
B4	F	34	60	2	18[n]	1.55	165*	23*	Symp	26.0	0.19[n]	NA	Sympathetic
B5	F	59	27	10	23[n]	1.20	137	12	Symp	5.0	0.16[n]	0	Sympathetic
B6	F	47	74	10	13	1.60	163	6	Symp[B]	2.0	0.01	0	Sympathetic predominant
B7	F	58	60	10	3	1.25	175*	37*	Symp	0.0	0.01	392	
B8	M	57	-12[n]	10	4	1.44	208[n]	184[n]	Both	0.0	0.02	C	Cholinergic
n, % normal			2, 25		4, 50	1, 13	2, 25	2, 25	1, 13	2, 25	5, 63	3, 43	

*Δ SBP/ TT, change in systolic blood pressure and time tolerated on head-up tilt; VR, Valsalva ratio; Sup. NA / ΔNA, supine noradrenaline and change on tilt; [r]repeat samples available; [n]within 5% normal cut off for age/sex,[34, 39, 80]; * noradrenaline levels not measured at time of biopsy, values from first autonomic testing performed at our unit, Symp/Parasymp, sympathetic/parasympathetic, C, catheter dependent, SR, HCT, spontaneous recovery, further stabilisation with hydrocortisone for Addison's disease, S>M: length-dependent, sensory>motor neuropathy, Bladder/SM: limited autonomic failure with bladder and sudomotor deficits; [B]Both sympathetic and parasympathetic pupillary deficits at initial assessment, only sympathetic deficits at time of recruitment to prospective study.*

4.3.10.4 Longitudinal biopsies after immune therapy

Four gAChR-positive and one gAChR-negative patients had repeat skin biopsies collected following immune therapy. Overall, the repeat biopsies showed improvements in IENF density and pilomotor innervation in parallel with the clinical recovery of the patients.

In the gAChR-positive group, one patient (patient 9) had limited clinical improvement with 3 cycles of plasma exchange and an adverse event to mycophenolate mofetil. She was therefore not on any active immunosuppression when repeat biopsies were collected. These showed a mixed picture, with some improvement at the thigh but deterioration at the leg (Table 4.8). Three patients (patients 6, 8 and 14) had a good clinical response to immunomodulatory treatment regimens (combinations of plasma exchange, prednisolone, mycophenolate and IVIg), with improvements in both somatic and autonomic innervation observed at both leg and thigh sites. Patient 8 had a relatively short disease duration at recruitment (4.8 years) and demonstrated marked clinical improvements with 2 cycles of plasma exchange and three cycles of IVIg given at 2g/kg. Repeat biopsies collected 0.9 years after baseline samples, two weeks after the third cycle of IVIg, showed a remarkable increase in intra-epidermal innervation in both leg and thigh, which were well within normal values for age/sex, with a highly branched pattern suggesting active regeneration (Figure 4.4). There were also marked improvements in the autonomic innervation of arrector pili muscles and sweat glands using with pan-neuronal, adrenergic and cholinergic markers (Figure 4.5).

When comparing the first and last biopsies available for the four gAChR-positive patients, after a range of 0.9-2.3 years, pilomotor innervation with adrenergic-marker DβH

improved significantly at the leg (mean change 15.9, 95% CI 8.9-22.8 f/mm, *P*=.02) and thigh (mean change 15.0, 95% CI 1.1-28.8 f/mm, *P*=.04), with two patients reaching normal values at the thigh (patient 8 and 14, Table 4.9). Pilomotor innervation with cholinergic marker VIP improved at the thigh (mean change 7.6, 95% CI 1.4-13.7 f/mm, *P*=.03).

One gAChR-negative patient (Patient B2) with sympathetic predominant autonomic failure had initial biopsies taken 4 months after disease onset which showed a severe loss of somatic and autonomic innervation. Repeat biopsies collected 4.5 months after treatment with rituximab showed marked increase in IENF, from 1.5 to 13.5 f/mm at the leg, from 1.6 to 19.3 f/mm at the thigh (both within normal values for age and sex), but very little change to the autonomic innervation.

Table 4.8. Longitudinal changes to cutaneous innervation following immune therapy

					Leg				Thigh			
						Mean PNF, f/mm				Mean PNF, f/mm		
Pt	Sex	Age, y	Disease duration, y	Treatment	IENF, f/mm	PGP	VIP	DBH	IENF, f/mm	PGP	VIP	DBH
6	F	50.9	4.3		1.5	25.9	20.1	15.5	0.7	28.7	12.1	16.1
		52.9	6.3	Pred, MMF	7.5	74.1[n]	43.5	NA	6.6	NA	4.9	NA
		53.1	6.5	IVIg	5.6	47.8	4.3	30.4	11.5	32.9	22.9	23.8
8	F	39.1	4.8		0.2	22.0	0.0	5.7	1.0	37.5	3.4	15.4
		40.0	5.7	PLEX, IVIg	15.2[n]	31.6	10.5	22.1	32.7[n]	37.9	9.3	36.5[n]
9	F	67.0	6.8		3.6	16.6	2.5	NA	1.6	15.0	4.3	6.5
		69.0	8.8	None*	2.1	11.2	3.8	0.8	5.8	36.4	15.0	13.7
14	F	54.5	33.7	PLEX	1.9	31.3	25.0	0.0	2.3	73.2[n]	16.1	19.9
		55.8	34.9	Pred	1.8	NA	0.0	0.0	2.1	36.7	10.0	14.8
		56.8	36.0	MMF	6.7	54.5	NA	NA	15.3	91.7[n]	19.0	43.7[n]
B2	M	42.1	0.3	PLEX	1.5	0.0	0.0	0.0	1.6	9.6	0.0	0.0
		42.5	0.7	Rituximab	13.5[n]	1.8	0.0	0.0	19.3[n]	NA	4.2	0.0

*Pred, prednisolone; MMF, mycophenolate mofetil; PLEX, plasma exchange; NA – no pilomotor muscles suitable for quantification, [n]within 5% normal cut off for age/sex,[34, 39, 80].*Had 3 exchanges of PLEX with limited clinical response, adverse reaction to MMF; [n]within 5% normal cut off for age/sex,[34, 39, 80]*

Table 4.9. Comparison between first and last biopsies in gAChR-positive patients

	Median, IQR		Mean change,	
	First	Last	95% CI	*P*-value
Age at biopsy, y	52.7, 47.9-57.7	55.0, 49.8-59.9		
Disease duration, y	5.8, 4.7-13.5	7.6, 6.3-15.6	1.8, 1.0-2.5	
IENF, f/mm				
Leg	1.7, 1.2-2.3	6.2, 4.7-8.8[1]	5.6, -5.3 to 16.5	.20
Thigh	1.3, 0.9-1.8	13.4, 10.1-19.7[1]	14.9, -3.8 to 33.6	.09
Pilomotor nerve fibre density, f/mm				
Distal leg				
PGP (N>67.2)	24.0, 20.7-27.3	39.7, 26.5-49.5	12.3, -8.9 to 33.5	.16
VIP (N>44.4)	13.8, 1.9-26.0	4.3, 4.1-7.4	-4.3, -49.8 to 41.2	.72
DBH (N>34.5)	5.7, 2.9-10.4	22.1, 11.5-26.3	15.9, 8.9-22.8	.02
Thigh				
PGP (N>60.7)	33.1, 25.3-46.3[1]	37.2, 35.5-51.4[1]	11.1, -5.4 to 27.6	.12
VIP (N>35.1)	8.2, 4.1-13.1	17.0, 13.6-20.0	7.6, 1.4-13.7	.03
DBH (N>34.5)	15.8, 13.2-17.1	30.2, 21.3-38.3[2]	15.0, 1.1-28.8	.04

[1,2] numbers of patients within normal range for age/sex.

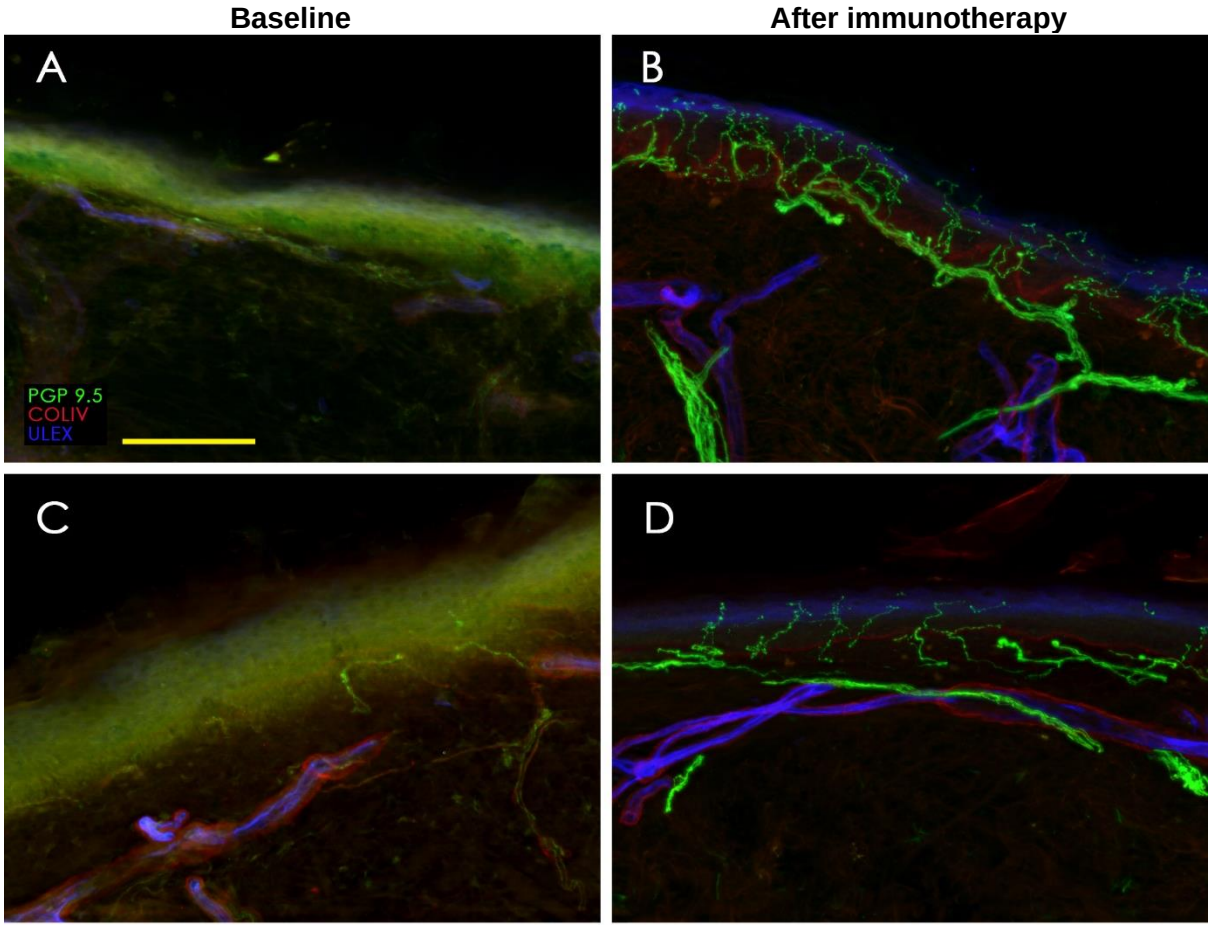


Figure 4.4. Baseline samples from the thigh (A) and distal leg (C) in a patient with gAChR-Ab-positive AAG (patient 6) prior to any immune therapy, and following 2 cycles of plasma exchange and 3 cycles of IVIg 2g/kg (B, D). There is a marked increase in the subepidermal plexuses and intra-epidermal innervation at both the thigh (B) and distal leg (D), with a highly branched pattern suggesting active regeneration.

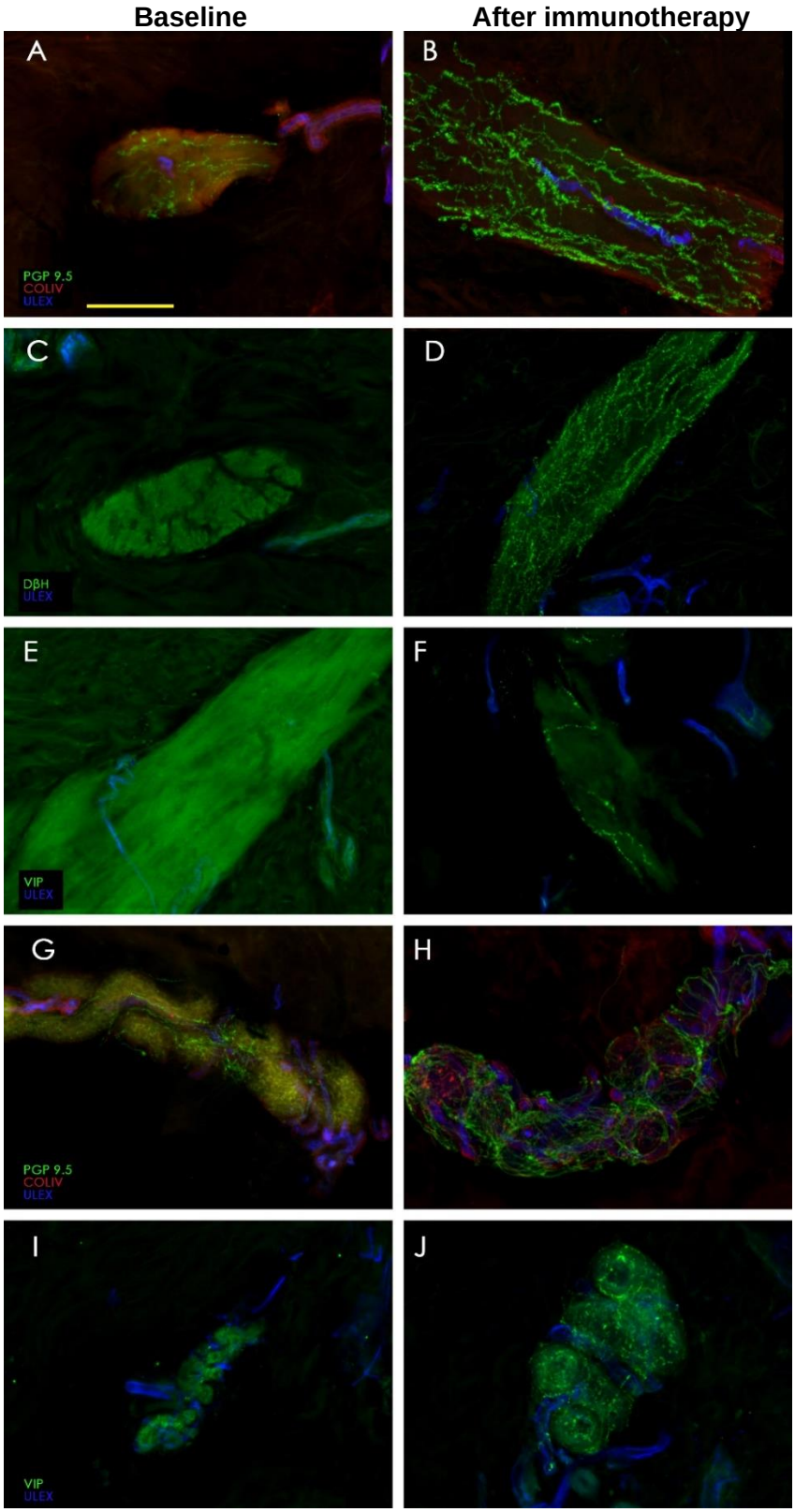


Figure 4.5. Autonomic innervation in gAChR-Ab-positive patients 6 (A, B, G, H, I, J) and 8 (C, D, E, F) at baseline and after immune therapy, with improvements in pilomotor innervation with pan-neuronal (B), adrenergic (D) and cholinergic (F) markers, and sudomotor innervation with pan-neuronal (H) and cholinergic (J) markers.

4.4 Discussion

This is the most comprehensive study to date retrospectively and prospectively comparing objective autonomic biomarkers in patients with subacute autoimmune autonomic failure, with and without the gAChR antibody, and PAF. In keeping with the observations from previous reports,[1, 27, 29, 30, 33] we confirmed that gAChR-positive patients had a distinct and consistent phenotype with severe widespread sympathetic and parasympathetic autonomic failure, with prominent cholinergic deficits across cardiovascular, pupillary, secretomotor, sudomotor and bladder testing, with pupil fatigue, typically non-length dependent post-ganglionic sudomotor dysfunction, and no significant peripheral somatic or central neurological deficits. In contrast, gAChR-negative patients had a more heterogenous phenotype, with most demonstrating predominantly sympathetic deficits, with relative preservation of parasympathetic cholinergic cardiovagal function, pupillary function and saliva production. Just over half had length-dependent postganglionic sudomotor dysfunction, and a few patients had a concurrent large fibre peripheral neuropathy/sensory neuronopathy. When trying to distinguish between gAChR-negative patients and PAF, a younger age at presentation, antecedent events, other autoimmune diseases, urinary retention requiring catheterisation, the presence of both parasympathetic and sympathetic pupillary deficits, and evidence for a large fibre neuropathy or neuronopathy were indicators of possible gAChR-negative autoimmune autonomic failure, whereas an older patient with RBD, supine hypertension, more severe orthostatic hypotension and impaired heart rate variability with deep breathing, with low supine noradrenaline levels was more likely to have PAF.

Skin biopsies showed a marked loss of intra-epidermal and pilomotor innervation in both gAChR-positive and gAChR-negative groups, with some gAChR-positive patients with a short duration of disease showed relatively preserved intra-epidermal innervation and pilomotor innervation with pan-neuronal marker PGP, but a severe loss of VIP-ir pilomotor fibres with relatively preserved DβH-ir fibres, suggesting a preferential loss of post-ganglionic cholinergic innervation. Sympathetic ganglia lie close to the spinal cord with long post-ganglionic neurons, whilst parasympathetic ganglia lie close to their target organs. It is possible that with immune attack against the autonomic ganglia, early post-ganglionic cholinergic denervation is observed before subsequent post-ganglionic adrenergic denervation patients with more longstanding disease. In contrast, some gAChR-negative patients with sympathetic predominant autonomic failure demonstrated more severe loss of DβH-ir pilomotor fibres, suggesting a different pathological process with preferential involvement of post-ganglionic adrenergic neurons. Overall, gAChR-negative patients had significantly lower adrenergic pilomotor innervation compared to gAChR-positive patients, in keeping with sympathetic predominant autonomic failure found in some of this cohort. An earlier study by Donadio at al studied 28 patients with autonomic neuropathies of multiple different aetiologies, and did not find a difference between the adrenergic and cholinergic innervation amongst the subgroups with sympathetic and parasympathetic predominant autonomic failure.[41] However, this study used patient symptoms to define the categories of sympathetic and parasympathetic predominant autonomic failure, included patients with autonomic neuropathies due to several different aetiologies, and used semi-quantitative methods to assess autonomic innervation. In contrast, our study used objective multimodal testing to determine to

characterise the autonomic phenotype of our patient group in detail and quantitative methods of assessing adrenergic and cholinergic autonomic nerve subpopulations.

We frequently observed that cutaneous nerves had a markedly irregular, beaded and fragmented appearance, amongst the gAChR-positive patients, as previously described,[1] but also in one of the gAChR-negative patients (B7). The clinical phenotype of this patient was not dissimilar to gAChR-positive patients, with pandysautonomia with no central or peripheral somatic deficits. This patient had positive anti-Ro antibodies in keeping with Sjogren's disease, which is associated with T-cell invasion of sensory and autonomic ganglia.[94] The abnormal fragmented appearance of cutaneous postganglionic nerves may reflect impaired axoplasmic transport distal to a more proximal, ganglionic pathology.

Two of the gAChR-negative patients (B1, B2) had biopsies collected within months of symptom onset that showed severe somatic and autonomic denervation. Both patients had a subacute onset of symptoms after an infectious antecedent event, with recovery after immune therapy. B1 had pandysautonomia, neuropathic pain, distal sensory loss with relatively preserved muscle strength, and a length-dependent sensory greater than axonal motor neuropathy on NCS/EMG. His skin biopsies showed virtually no subepidermal plexuses and a complete loss of intra-epidermal nerves and severe autonomic denervation. In contrast, B2 had severe sympathetic predominant autonomic failure, with no evidence of a small or large fibre peripheral somatic neuropathy on neurophysiological testing. His biopsies showed a severe loss of subepidermal plexuses, with almost complete loss of intra-epidermal nerves, with severe denervation to the autonomic adnexa. Repeat biopsies collected after 4.5 months of rituximab treatment,

when we had started to observe some improvements on clinical testing, showed a dramatic recovery of intra-epidermal innervation to within normal values at both the leg and thigh, with limited recovery of the autonomic nerves at this early stage. It is possible that the primary immune target in this case was the autonomic nerves, with loss of intra-epidermal innervation occurring as a secondary process, and improving rapidly after commencing immune treatment, prior to the recovery of the autonomic nerves.

Previous case series have described gAChR-negative patients with concurrent sensory symptoms and signs and abnormal NCS/EMG.[27, 33] Our gAChR-negative cohort had three patients with abnormal NCS/EMG, with heterogenous phenotypes. The first patient, B1, with antecedent gastrointestinal symptoms and severe pandysautonomia with a length-dependent axonal sensorimotor neuropathy, elevated CSF protein and good recovery months after steroids and IVIg, could be argued to fall within the spectrum of an axonal variant of GBS, with prominent autonomic involvement. Autonomic involvement is a recognised feature of GBS and can occur in up to two-thirds of patients, including brady- and tachyarrhythmias, blood pressure variability, including orthostatic hypotension, sudden cardiac arrests, gastrointestinal and genitourinary symptoms.[95] The second patient with a meningoencephalitis, severe sympathetic predominant autonomic failure, sensory neuropathy and CSF GFAP antibodies, with an excellent response to intravenous and oral steroids, was the only patient within our cohort to demonstrate both central and peripheral somatic involvement. CSF GFAP antibodies are associated with a monophasic, immunotherapy responsive CNS disease, with previous series reporting autonomic dysfunction reported in 24-63% of cases, although the nature of autonomic dysfunction has not previously been deeply characterised.[96-98] The third patient with anti-

Ro antibodies, sicca syndrome, abnormal Schirmer's testing and a sensory neuronopathy fulfilled criteria for Sjogren's syndrome. A large clinico-pathological series of 92 patients with primary Sjogren's syndrome associated neuropathy found autonomic symptoms were widely present in the cohort, but particularly prominent in three patients, suggesting while autonomic involvement in Sjogren's syndrome is typically mild, severe autonomic neuropathy lies within the spectrum of Sjogren's syndrome related neuropathies.[94] These heterogenous cases highlight that within the gAChR-negative cohort, there are likely to be different diseases presenting with subacute autonomic failure, with and without central and peripheral somatic deficits. Deep characterisation of their autonomic and other neurological deficits should help stratify this diverse cohort into groups with distinct clinical phenotypes, allowing more targeted investigations for the underlying aetiology and treatment strategies.

Goldstein et al described a gAChR-negative young woman presenting with acute pandysautonomia with severe gastrointestinal dysmotility requiring a jejunostomy, suggesting prominent cholinergic autonomic failure. By the time she was referred to a tertiary autonomic centre, her gastrointestinal symptoms had resolved and investigations showed sympathetic predominant cardiovascular and pupillary deficits, with complete loss of pilomotor fibres, decreased cholinergic sudomotor innervation, and moderately severe intra-epidermal nerve fibre loss. The authors speculate the target of immune attack was the post-ganglionic unmyelinated axons, with preferential recovery of parasympathetic neurons and persistent sympathetic denervation. This case is very similar to one of our gAChR-negative cases (patient B6), who presented in her 20s with severe pandysautonomia with prominent gastrointestinal symptoms and combined

sympathetic and parasympathetic pupillary deficits at first assessment, that improved over time, leaving a sympathetic predominant autonomic failure and bilateral Horner's syndrome at the time of recruitment to the prospective study. Her skin biopsies also showed a complete loss of pilomotor innervation and fairly severe loss of intra-epidermal innervation. Within our heterogenous group of gAChR-negative patients, we have seen that there are some patients with a clinical phenotype similar to the gAChR-positive group, but others with more sympathetic deficits, some with central and /or peripheral somatic involvement, with varying degrees of postganglionic dysfunction and denervation on clinical testing and punch skin biopsies. The gAChR-Ab negative group is likely represent a mixture of different pathologies, with central, ganglionic and post-ganglionic involvement.

4.4.3 Limitations

This study was conducted at a national autonomic referral centre, with in-depth cardiovascular, sudomotor, secretomotor, pupillary and bladder testing performed on site. Gastro-intestinal studies are not routinely performed on site and not systematically studied. Most patients referred to our centre had orthostatic intolerance, and few patients with isolated cholinergic failure, like patient B8, presenting with predominant bladder, bowel, sweating, secretomotor symptoms, with a relative paucity of cardiovascular symptoms, who may be referred to gastroenterologists rather than to our centre. The COVID-19 pandemic occurred halfway through our prospective study, impacting on clinical services. Some patients living a long distance from our centre were reviewed remotely rather than in-person, so we were not able to collect follow up biopsies at regular intervals following immune therapy as originally planned. Due to the pandemic,

there was also more reservation from treating clinicians and patients about initiating a trial of immune therapy for gAChR-negative patients, especially in patients with more longstanding disease where it was unclear whether a benefit would be seen. As a result, we had fewer follow up skin biopsies from gAChR-negative patients to include in the study.

4.4.4 Summary and future directions

Our study has defined the clinical features and multi-modal cardiovascular, neurohormonal, pupillary, secretomotor, sudomotor and cutaneous biomarkers that differentiate between PAF and gAChR-positive and negative autoimmune autonomic failure. Patients with gAChR-negative autoimmune autonomic failure had greater loss of cutaneous adrenergic fibres, in keeping with the sympathetic predominant autonomic failure seen in some of the cohort, which were relatively preserved in gAChR-positive patients with early disease. Recovery on longitudinal biopsies after immune treatment correlated with clinical recovery and autonomic phenotype. Skin biopsies are promising, minimally invasive method of study patterns of peripheral nerve degeneration and recovery should be explored in other acquired and inherited neuropathies like CIDP and aTTRm. Given the heterogeneity in the clinical phenotype of gAChR-negative patients, all patients should have an in-depth assessment with multi-modal autonomic biomarkers at baseline to define their deficits, including investigations for specific diseases associated with autoimmune autonomic failure depending on clinical phenotype, and have an individualised plan for monitoring for objective improvements with immune therapy.

Chapter 5. Multimodal autonomic and cutaneous biomarkers in α-synucleinopathies

5.1 Introduction

Having studied multiple autonomic biomarkers in patients with autoimmune autonomic failure, I aimed to evaluate whether a panel of clinical autonomic biomarkers could help distinguish between patients with primary chronic autonomic failure due to neurodegenerative diseases characterised by the deposition of α-synuclein.

5.1.1 Alpha-synycleinopathies

Alpha-synucleinopathies are a group of neurodegenerative diseases characterised by the deposition of abnormally phosphorylated α-synuclein within the central and peripheral nervous system, with varying clinical manifestations, including autonomic failure. They include PAF, MSA, PD, and DLB. PAF classically presents in mid to later life with orthostatic hypotension in the context of more widespread autonomic failure, in the absence of other neurological features.[14] MSA is characterised by autonomic failure, commonly with severe genitourinary involvement, poorly-levodopa-responsive parkinsonism, cerebellar, and corticospinal features, and is associated with more rapid disease progression and poor survival.[20, 51, 99] PD is characterised by a levodopa-responsive motor syndrome of bradykinesia, with rest tremor and rigidity, but non-motor manifestations including autonomic dysfunction are commonly seen and can be severe.[53] DLB presents with progressive cognitive decline with prominent deficits in attention, executive function, and visuoperceptual ability, fluctuating cognition, recurrent visual

hallucinations, RBD and parkinsonism.[54] Lewy body disease or LBD is a term used to describe both PD and DLB.

Neuropathologically, PAF, PD and DLB are characterised by the accumulation of misfolded α-synuclein in neuronal cytoplasmic inclusions, called Lewy bodies, whereas in MSA, α-synuclein is primarily deposited in glial cells. Patients with PAF have predominantly peripheral deposition of α-synuclein, with associated postganglionic autonomic denervation.[10] Although central α-synuclein deposition is also observed, central dopaminergic neurones are typically preserved, in contrast to patients with PD and MSA, which may explain the lack of central neurological features. Plasma noradrenaline is a measure of peripheral sympathetic neural activity. In both diseases, plasma noradrenaline fails to rise with orthostasis, but supine noradrenaline levels are typically low in PAF, suggesting postganglionic dysfunction, but normal in MSA, suggesting primarily pre-ganglionic dysfunction of the autonomic nervous system.[14, 51, 59] Similarly, cardiac uptake of adrenergic analogue MIBG tends to be intact in MSA,[100] in contrast to PAF and the other Lewy body diseases, although more recent studies suggest it can be impaired in up to 30% of patients with MSA.[101] Patients with MSA also classically demonstrate a preganglionic pattern of anhidrosis,[99] but again more recent, larger studies have shown that post-ganglionic sudomotor dysfunction and denervation also occurs in MSA,[102-104] with increasing frequency over time. Overall, these studies suggest that within the α-synucleinopathies, the autonomic nervous system can be affected in several domains and at different levels from central networks within the brain through to the autonomic ganglia and post-ganglionic neurons.

5.1.2 Natural history of PAF and phenoconversion to MSA and LBD

Previous natural history studies have shown that 12-34% of patients with PAF eventually develop progressive motor and/or cognitive symptoms and fulfil criteria for MSA, PD or DLB.[15-17] Clinical features and biomarkers that have been reported to predict future conversion to MSA include a higher resting heart rate, greater heart rate rise on tilt, higher Valsalva ratio, higher supine noradrenaline, normal cardiac MIBG, preserved olfaction, and subtle motor signs not qualifying for parkinsonism or ataxia at initial assessment.[15-18] Retrospective and prospective studies have identified early severe bladder symptoms and catheterisation as risk factors for conversion to MSA,[16-18] but have not systematically studied patients with formal bladder assessments. Recently, the Movement Disorders Society have introduced a research category of possible prodromal MSA including patients with isolated autonomic failure with subtle motor signs.[20]

We hypothesised that deep phenotyping with multimodal biomarkers might reveal characteristic autonomic profiles and insights about the predominant site of involvement of the autonomic nervous system in the different α-synucleinopathies. Characteristic autonomic phenotypes could help confirm the underlying diagnosis in patients presenting with early disease where the aetiology is unclear.

5.1.3 Aims of study

I aimed to deeply characterise the autonomic phenotype in a large group of patients with α-synucleinopathies referred to a single national autonomic unit using a comprehensive protocol of autonomic testing across multiple domains to try to identify characteristic autonomic phenotypes that could help distinguish between the different α-synucleinopathies at an early stage of the disease. A secondary aim was to identify

biomarkers that could help to predict future phenoconversion from patients presenting with PAF to MSA or LBD.

5.2 Methods

5.2.1 Overall study design

We performed two linked studies. Firstly, we retrospectively compared the cardiovascular testing, plasma catecholamines and pupillometry at first evaluation of 391 patients seen at a national autonomic referral centre between 1987-2021 with a final clinical diagnosis of PAF, MSA, and LBD according to established consensus criteria at their most recent clinical review.[14, 20, 53, 54] Secondly, we prospectively recruited 52 patients with the above diagnoses between 2018-2021 and studied them with a multimodal testing protocol including the investigations above, as well as bladder studies, dynamic sweat testing, and questionnaires to assess their autonomic symptoms and quality of life.

5.2.1.1 Retrospective study

719 patients referred to our national autonomic referral centre with suspected autonomic failure between 1987-2021 were considered for inclusion. 328 patients were excluded, including patients with hereditary or acquired amyloidosis (181), autoimmune autonomic failure (52), autonomic failure due to diabetes mellitus, chemotherapy, or radiotherapy (21), other atypical Parkinsonian and cerebellar syndromes (15), other rare genetic diseases (12), other miscellaneous autonomic disorders e.g., afferent baroreflex disorder, Ross or Harlequin syndrome (26), and patients where the diagnosis was unclear at time of analysis (21). 391 patients with a final diagnosis of an α-synucleinopathy, including patients with PAF, MSA, and LBD (including patients with PD and DLB) according to established diagnostic criteria [14, 52-54] at the time of their most

recent clinical review, up to July 2021, or on post-mortem analysis if available were included.

As part of their clinical work up, all patients underwent a comprehensive clinical evaluation and examination, cardiovascular autonomic testing, plasma catecholamines, as well as screening for potential secondary causes of autonomic failure, imaging, neurophysiology, with further opinions from colleagues with expertise in Movement Disorders, Peripheral Nerve, Neuro-ophthalmology and Uro-neurology as required, followed by a multi-disciplinary team discussion regarding the likely aetiology of autonomic failure based on the initial assessments. All patients had regular follow-up assessments to monitor disease progression and screen for development of additional features, allowing greater refinement of their clinical diagnoses over time.

Data was retrospectively extracted from first available cardiovascular autonomic testing, plasma catecholamines and pupillometry.

5.2.1.2 Prospective study

103 patients were prospectively recruited to a natural history study from April 2018 to June 2021 and underwent detailed multimodal autonomic testing including cardiovascular autonomic testing, plasma catecholamines, dynamic sweat testing, bladder studies, and standardised autonomic symptom and quality of life questionnaires. We included the 52 patients that had a final diagnosis of an α-synucleinopathy, excluding 51 patients with autoimmune autonomic failure, other inherited and acquired diseases, and those where the diagnosis was unclear at the time of final analysis.

Both studies were approved by the local ethics committee and health research authority. All prospectively recruited patients provided written informed consent according to the Declaration of Helsinki.

5.2.2 Cardiovascular autonomic testing and plasma catecholamines

All patients underwent cardiovascular autonomic testing as previously described[55] with beat-to-beat recordings of blood pressure and heart rate at rest in the resting supine position and with:

5) Active standing challenge at 1-, 3- and 5 minutes
6) Passive head up tilt to 60° for up to 10 minutes
7) Isometric exercise (sustained handgrip for 3 minutes at a third of maximum voluntary contraction pressure)
8) Deep breathing, at a rate of 6 breaths/min
9) Valsalva manoeuvre (forced expiration at 40mmHg for 10 seconds)

Patients underwent an active stand for up to 5 minutes and were passively tilted for a maximum of 10 minutes. Some patients developed severe orthostatic hypotension with signs of cerebral hypoperfusion on orthostasis necessitating early termination of stand and/or tilt. The OIR-stand was calculated by dividing the fall in systolic blood pressure (ΔSBP) over the time tolerated on stand in minutes, up to a maximum of 5 minutes. The OIR-tilt was calculated by dividing the ΔSBP in mmHg over the time tolerated on head-up tilt in minutes, up to a maximum of 10 minutes.

Valsalva ratio (VR) was calculated by dividing maximum heart rate developing during Phase II of the Valsalva manoeuvre over the minimum heart rate occurring within 30

seconds of the peak heart rate. PRT was defined as the time taken for the systolic blood pressure to recover from Phase III back to the baseline.[56]

Blood samples were collected via intravenous forearm catheter at rest and following orthostasis for analysis of plasma noradrenaline using high performance liquid chromatography.

5.2.2 Pupillometry

Infrared pupillometry was used to record baseline pupil diameters and responses to stimulation with light impulses and pharmacological stimulation with topical agents including 0.5% apraclonidine, 1% hydroxyamphetamine, 4% cocaine and 0.125% pilocarpine. Absent or diminished pupillary light reflexes and supersensitivity to dilute pilocarpine were indicative of parasympathetic deficits, and delayed pupillary redilation following a light impulse, diminished response to cocaine or supersensitivity to apraclonidine indicated sympathetic deficits. From May 2019, patients were also examined with a prolonged light stimulus to assess for pupillary fatigue, a unique phenomenon previously reported only in patients with seropositive autoimmune autonomic ganglionopathy.[31]

5.2.3 Sudomotor testing

Patients underwent DST at the distal leg and forearm, an assessment of postganglionic sudomotor function.[63] After iontophoresis with 1% pilocarpine, skin was coated with iodine and formation of sweat gland imprints on starch covered tape was recorded. Density of activated sweat glands/cm^3, sweat output/min/cm^3, and average sweat output/gland was calculated for each site and the mean value for both sides used.

5.2.4 Urinary studies

Urinary flow when voiding with the sensation of a full bladder was assessed by uroflowmetry (Albany Medical SmartFlow) and post-void residual volume measured using a bladder ultrasound scanner (Bardscan Realtime).

5.2.5 Patient reported outcomes

Patient reported outcomes were collected using the abbreviated and refined composite autonomic symptom score (COMPASS-31), the small fibre neuropathy symptom inventory questionnaire (SFN-SIQ), and the 36-item short form health survey (SF-36). The COMPASS-31 assesses patient symptoms in six autonomic domains, with weighted subscores for orthostatic intolerance, vasomotor, secretomotor, gastrointestinal, bladder, and pupillomotor symptoms, giving an overall autonomic symptom score from 0 to 100.[66] The SFN-SIQ assesses 12 sensory and autonomic symptoms including changes in sweating, diarrhea, constipation, micturition problems, dry eyes, dry mouth, orthostatic dizziness, palpitations, flushes, skin sensitivity, burning, restless legs, and sheet intolerance of legs, with patients asked to provide a score of 0 (never) to 3 (always) for each section.[67] The SF-36 assesses eight health concepts, 1) physical limitations due to health problems, 2) social limitations due to physical or emotional problems, 3) role limitations due to physical health problems, 4) bodily pain, 5) general mental health (psychological distress and well-being), 6) role limitations due to emotional problems, 7) vitality (energy and fatigue), and 8) general health perceptions, with a scoring algorithm generating a possible range of 0 (worst possible health) to 100 (best possible health) for each of the eight domains.[68]

5.2.6 Statistical analysis

Data were captured electronically using a secure Research Electronic Data Capture (REDCap) platform. Statistical analysis was performed using R Studio, Version 1.2.1335. Summary data has been displayed as median, interquartile range for continuous data and numbers, percentages for categorical data. Distributions of data were assessed for normality by visual inspection and using Shapiro-Wilk tests. Pairwise comparisons were made with unpaired two-tailed T-tests/Wilcoxon rank-sum tests and group comparisons were made with ANOVA/Kruskal-Wallis tests with post-hoc comparisons using Tukey/Dunn's tests with Bonferroni corrections as appropriate. Chi-squared tests were used to compare categorical data. Spearman's rank correlation was used to assess the correlation between linear variables. $P<.05$ was considered significant.

5.2.6.1 Univariate and multivariate logistic regression

Variables that were significantly different between groups were assessed in a univariate logistic regression model to determine if they were significant predictors of a particular final diagnosis. Variables that were significant predictors at a univariate level were used in a multivariate model, after removing significantly correlated variables ($\rho>0.40$ on Spearman's rank correlation). For correlated variables, the variable with the lowest *P*-value on univariate analysis was selected for use in multivariate analysis.

5.3 Results

5.3.1 Retrospective patient cohort

The retrospective cohort included 391 patients diagnosed with an α-synucleinopathy by their final review: 146 patients with PAF, 157 with MSA (79 with predominantly cerebellar features, 65 with predominantly Parkinsonian features, and 13 with mixed features) and

88 with LBD (55 with PD and 33 with DLB). The cohort included 47 patients with neuropathologically established MSA, confirmed at post-mortem at the Queen Square Brain Bank.[105]

At first assessment, patients with MSA were significantly younger (median age 60 [IQR 53-66] years), compared to patients with PAF (68 [59-75] years) and LBD (71 [65-75] years) (*P*=4.9e-16). A greater proportion of the LBD group were male compared to the PAF group (68% v 51%, *P*=.02) (Table 5.1).

Patients with PAF had significantly more severe orthostatic hypotension compared to patients with MSA and LBD, with the largest ΔSBP on tilt (71 [55-90] mmHg vs 48 [26-72] mmHg (MSA) and 49 [31-79] mmHg (LBD) (*P*=3.7e-10) and OIR-tilt (8.9 [6.1-17.1] vs 4.8 [2.6-8.2] in MSA and 5.5 [3.1-11] in LBD groups, *P*=2.3e-12. They also had significantly lower Valsalva ratio (1.15 [1.07-1.32] vs 1.24 [1.13-1.43] in MSA and 1.23 [1.14-1.37] in LBD, *P*=.001 and heart rate increment with isometric exercise (3 [1-7] bpm vs 4 [2-9] bpm in MSA and 5 [2-9] in LBD groups, *P*=.003), as well as lower heart rate variability with deep breathing (HR_{DB}), although this was not significant at the 5% level (3 [0-7] bpm vs 4 [1-7] bpm in MSA and 5 [1-9] bpm in LBD, *P*=.05). Patients with PAF had significantly lower supine noradrenaline levels (176 [142-204] pg/ml) compared to patients with LBD (223 [202-254] pg/ml) and MSA (263 [222-308] pg/ml), *P*<2.2e-16. Orthostatic increment of noradrenaline was minimal in all groups, with no significant difference between groups.

71% (41/58) of the PAF patients had abnormal pupillary function, compared only 32% (8/25) of MSA patients and 20% (1/10) of LBD patients, *P*=.0003, with sympathetic deficits being most common abnormality in all groups. None of the patients had

combined sympathetic and parasympathetic deficits. None of the 13 patients (11 PAF, 2 MSA) who had dedicated testing to look for pupil fatigue had this finding, a characteristic finding in patients with gAChR-Ab positive AAG, as described in Chapters 3 and 4.

Table 5.10. Comparison of cardiovascular, neurohormonal, and pupillary autonomic biomarkers at first assessment in 391 patients with PAF, MSA and LBD

	Median, IQR			P-value			
	PAF, n=146	MSA, n=157	LBD, n=88	ANOVA	PAF vs MSA	PAF vs LBD	MSA vs LBD
Age, years	68, 59-75	60, 53-66	71, 65-75	5 e-16	2 e-08	.01	5 e-15
Male sex, n, %	75, 51	93, 58	63, 68	.04	.25	.02	.19
Cardiovascular, n	146	157	88				
Supine							
SBP, mmHg	152, 136-173	141, 123-141	147, 126-168	.001	.001	.18	.60
HR, bpm	67, 60-73	73, 64-81	65, 59-72	1 e-07	8 e-6	.73	4 e-6
Head up tilt							
ΔSBP, mmHg	71, 55-90	48, 26-72	49, 31-79	4 e-10	1 e-9	2 e-05	1
ΔHR, bpm	9, 3-17	11, 5-18	11, 7-18	0.23			
OIR - tilt	8.9, 6.1-17.1	4.8, 2.6-8.2	5.5, 3.1-11	2 e-12	2 e-12	3 e-05	.24
Iso. exercise							
ΔSBP, mmHg	3, -4-9	4, -2-9	6, 1-13	.02	1	.01	.08
ΔHR, bpm	3, 1-7	4, 2-9	5, 2-9	.003	.01	.02	1
HR_{DB}, bpm	3, 0-7	4, 1-7	5, 1-9	.05			
Valsalva ratio	1.15, 1.07-1.32	1.24, 1.13-1.43	1.23, 1.14-1.37	.001	.002	.04	1
Noradrenaline, n	135	118	77				
Supine NA, pg/ml	176, 142-204	263, 222-308	223, 202-254	<2 e-16	5 e-28	5 e-09	.001
Δ NA with tilt	10, 2-21	11, 3-45	15, 4-45	.05			
Pupillometry, n	58	25	10				
Normal	17, 29	17, 68	8, 80	.0003	.002	.004	.69
Parasympathetic	4, 7	1, 4	0, 0	1			
Sympathetic	37, 64	7, 28	2, 20	.002	.01	.01	1

SBP, systolic blood pressure, HR, heart rate; NA, noradrenaline.

5.3.2 Summary of cardiovascular, neurohormonal, and pupillary profiles at first evaluation of patients with PAF, MSA and LBD

Overall, compared to the MSA and LBD groups, patients with PAF had significantly more impaired sympathetic cardiovascular and pupillary function and reduced supine noradrenaline levels at first evaluation, in keeping with postganglionic adrenergic denervation (Table 5.2). Compared to the PAF and LBD groups, patients with MSA were significantly younger at first evaluation, in keeping with a more aggressive pathology,

with higher supine heart rate and supine noradrenaline levels, in keeping with relatively preserved postganglionic adrenergic innervation. The LBD patients were significantly older than both other groups at first presentation, in keeping with a more gradually progressive neurodegenerative disorder, with intermediate supine noradrenaline levels that were higher than PAF patients but lower than MSA patients.

Table 5.11. Summary of significant differences between clinical features, cardiovascular testing, noradrenaline and pupillometry in PAF, MSA and LBD groups at first evaluation.

First evaluation	PAF	MSA	LBD
Age <70 years	+++	+++++	++
Supine heart rate ≥ 70bpm	++	+++	++
Δ SBP on tilt ≥ 60 mmHg	++++	++	++
OIR-tilt ≥ 6	++++	++	+++
Valsalva ratio < 1.2	++++	++	+++
Supine noradrenaline ≥ 200pg/ml	++	+++++	++++
Pupillometry			
Normal	++	++++	++++
Sympathetic deficit	++++	++	+
Parasympathetic deficit	+	+	-

+, 1-20%;
++, 21-40%;
+++, 41-60%;
++++, 61-80%;
+++++, 81-100%.

5.3.3 Univariate and multivariate logistic regression modelling

5.3.3.1 Variables on initial assessment associated with a final diagnosis of MSA versus PAF

In a univariate logistic regression model, younger age at first assessment, lower supine SBP, higher supine heart rate, lower ΔSBP on tilt and OIR-tilt, higher ΔHR on isometric exercise, higher Valsalva ratio, higher supine noradrenaline levels and normal pupils

were predictive of final diagnosis of MSA rather than PAF. Continuous variables were checked for correlations prior to multivariate logistic regression analysis. For correlated variables, the variable with lowest *P*-value on univariate analysis was used for multivariate analysis. OIR-tilt correlated strongly with ΔSBP on tilt (ρ=0.88) as expected and moderately with supine SBP (ρ=0.41). Assessments of HR variability were also correlated: Valsalva ratio correlated with HRDB (ρ=0.47) and ΔHR with isometric exercise (ρ=0.47).

On multivariate analysis, significant predictors for a final diagnosis of MSA rather than PAF were having supine noradrenaline ≥ 200pg/ml at first assessment (OR 20, 95%CI 2-158, *P*=.004), normal pupils (OR 17, 95%CI 2-188, *P*=.02), Valsalva ratio≥1.2 (OR 7.5, 95%CI 1.3-44.1 *P*=.03), and higher supine heart rate (OR 1.4, 95% CI 1.1-1.7, *P*=.01) (Table 5.12).

5.3.3.2 Variables on initial assessment associated with a final diagnosis of LBD versus PAF

Univariate modelling showed older age, male sex, lower supine SBP, lower ΔSBP and OIR on head-up tilt, higher ΔHR with isometric exercise, higher Valsalva ratio, higher HRDB, higher supine noradrenaline and normal pupils were predictive of final diagnosis of LBD rather than PAF. There were only 10 LBD patients with pupillometry data, so this was not included in multivariate modelling for the LBD group. Excluding pupillometry in a multivariate model, significant predictors for a final diagnosis of LBD rather than PAF were supine noradrenaline≥200pg/ml (OR 5.50, 95%CI 2.89-10.47, *P*=2.1e-07), OIR<6 (OR 3.26, 95%CI 1.76-6.04, *P*=.0002), male sex (OR 3.10, 95%CI 1.31-7.30, *P*=.01) and older age at first assessment (OR 1.07, 95%CI 1.03-1.12, *P*=.002).

5.3.3.3 Variables on initial assessment associated with a final diagnosis of MSA versus LBD

Univariate and multivariate modelling showed age<70years at first assessment (OR 10.5, 4.8-22.9, *P*=4.1e-9), supine HR≥70bpm (OR 2.6, 1.5-4.3, *P*=.0005), and higher supine noradrenaline (OR 1.01, 1.00-1.02, *P*=.001) were all significant predictors of a final diagnosis of MSA rather than LBD. Significant predictors from initial assessment on multivariate regression modelling are summarised in Table 5.3 and Figure 5.1.

Table 5.12. Variables on initial assessment associated with significantly increased odds of a final diagnosis on multivariate logistic regression modelling for A) MSA versus PAF, B) LBD versus PAF, C) MSA versus LBD

Variables on initial assessment	OR, 95% CI	P-value
A) MSA versus PAF		
Supine noradrenaline ≥ 200pg/ml	20, 3-158	.004
Normal pupils	17, 2-188	.02
Valsalva ratio ≥1.2	7.4, 1.3-44.2	.03
Supine heart rate, bpm	1.3, 1.1-1.7	.01
B) LBD versus PAF		
Supine noradrenaline ≥ 200pg/ml	5.5, 2.9-10.5	2.1e-07
OIR-tilt < 6	3.3, 1.8-6.0	.0002
Male sex	3.1, 1.3-7.3	.01
Age, years	1.07, 1.03-1.12	.002
C) MSA versus LBD		
Age<70 years	10.5, 4.8-22.9	4.1e-09
Supine heart rate≥70bpm	2.6, 1.5-4.4	.0005
Supine noradrenaline, pg/ml	1.01, 1.00-1.02	.001

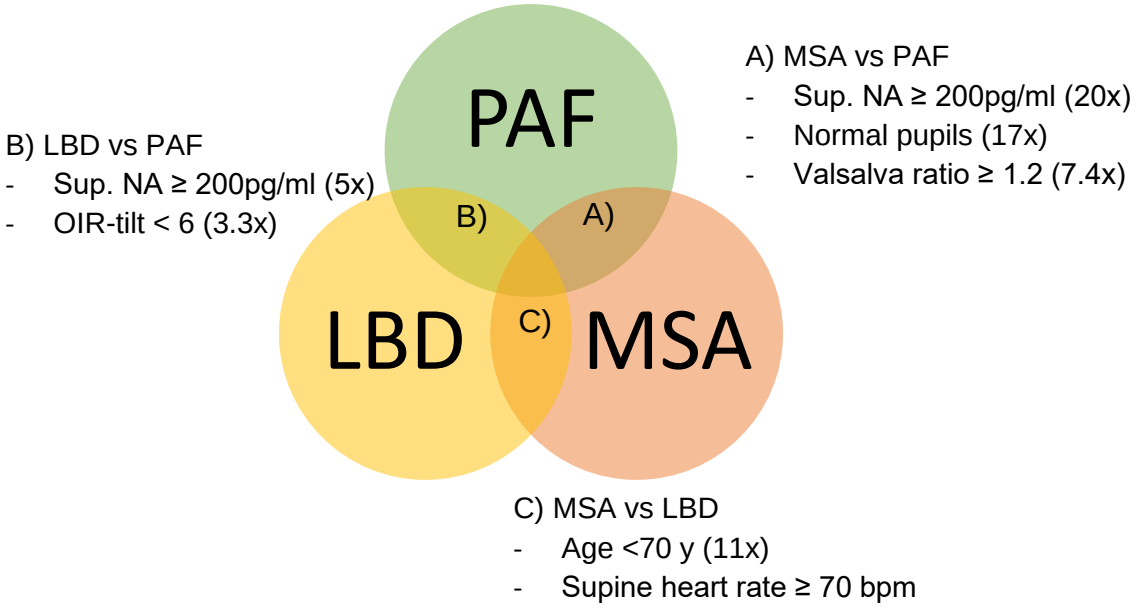


Figure 5.1. Variables on initial autonomic assessment associated with a significantly increased odds ratio of a final diagnosis of A) MSA rather than PAF B) LBD rather than PAF and C) MSA rather than PAF on multivariate logistic regression modelling. NA, noradrenaline

5.3.4 Phenoconversion from PAF to MSA or LBD

Based on these initial analyses, it appeared that several biomarkers on objective autonomic testing at initial assessment differed between patients with a final diagnosis of PAF, MSA and LBD. This entire retrospective cohort of patients with a final diagnosis of an α-synucleinopathy included patients with parkinsonian and/or cerebellar signs when they were first referred, as well as patients with symptoms of isolated autonomic failure for at least 3 years, with no or only subtle motor features that were difficult to discern from normal variants or aging and did not meet criteria for parkinsonism or a cerebellar syndrome, in other words, meeting a diagnosis of PAF at initial assessment.

We looked at the subset of 194 patients in our cohort who fulfilled criteria for a diagnosis of PAF at their initial assessment, to see if the autonomic phenotype at first assessment differed between those who later phenoconverted to MSA or LBD and those who

retained a diagnosis of PAF at their final clinical review. At their final review, 74% (144 patients) retained a diagnosis of PAF, and 26% (50 patients) had developed signs and symptoms fulfilling the criteria for a more widespread α-synucleinopathy: 32 with LBD, 18 with MSA.

Table 5.4. Comparison between patients with stable PAF and patients phenoconverting to MSA or LBD

	Median, IQR		*P*-value
	Stable PAF, n=144	Phenoconverter, n=50	
Age, years	68, 59-75	67, 60-73	.59
Male sex, n, %	73, 51	37, 73	.01
Cardiovascular testing, n	144	50	
Supine			
SBP, mmHg	152, 136-173	141, 132-162	.06
HR, bpm	67, 60-74	67, 62-74	.58
Head up tilt			
ΔSBP, mmHg	71, 56-90	62, 45- 86	.09
ΔHR, bpm	10, 3-18	13, 8-21	.045
OIR - tilt	9, 6.2-17.5	6.3, 4.5-11.6	.003
Isometric exercise			
ΔSBP, mmHg	3, -4 to 9	6, 2-13	.01
ΔHR, bpm	3, 1-7	4, 2-8	.22
HR_{DB}, bpm	3, 0-6.5	4, 1-11	.04
Valsalva ratio	1.15, 1.07- 1.32	1.21, 1.16-1.35	.06
Noradrenaline, n	134	48	
Supine NA, pg/ml	176, 141-204	210, 161-246	.0002
ΔNA on tilt, pg/ml	10, 2-22	9, 2-28	.97
Pupillometry, n	57	15	
Normal	17, 30	10, 67	.02
Sympathetic	36, 63	5, 33	.046

SBP, systolic blood pressure; HR, heart rate; NA, noradrenaline.

5.3.4.1 Predictors of phenoconversion

Amongst the patients presenting initially with PAF, the phenoconverter group had proportionally more males compared to the stable PAF group (73% vs 54%, *P*=.01). On initial assessment, compared to patients with stable PAF, patients who phenoconverted had less severe orthostatic hypotension on tilt, (median OIR-tilt 6.3 [IQR 4.5-11.6] vs 9 [6.2-17.5], *P*=.004), more preserved HR_{DB} (4 [1-11] bpm vs 3 [0-7] bpm, *P*=.04), supine noradrenaline (210 [161-246] pg/ml vs 176 [141-204] pg/ml, *P*=.0002) and more preserved pupillary function (67% vs 30% with normal pupils, *P*=.02).

On univariate logistic regression modelling, normal pupils (OR 4.7, 95% CI 1.2-15.9, *P*=.01), HR_{DB}≥10bpm (OR 2.9, 95% CI 1.5-5.4, *P*=.001), male sex (OR 2.6, 95% CI 1.3-5.1, *P*=.01), supine noradrenaline≥200pg/ml (2.4, 95% CI 1.5-3.9, *P*=.0002), and OIR<6 (OR 1.89, 95% CI 1.17-3.04, *P*=.01) were significant predictors of phenoconversion. A multivariate analysis was not performed due to the small numbers of converters with all parameters assessed.

5.3.4.2 Predictors of conversion to MSA versus LBD

Patients converting to MSA were younger at first assessment than those converting to LBD (59 [50-67] years vs 71 [64-74] years, *P*=.001), with higher supine noradrenaline (242 [201-316] pg/ml vs 204 [159-228] pg/ml, *P*=.04. On univariate and multivariate analysis, younger age at first assessment (OR 0.88, 95% CI 0.81-0.97, *P*=.01) and higher supine noradrenaline (OR 1.01, 95% CI 1.00-1.03, *P*=.04) were predictors of phenoconversion to MSA rather than LBD.

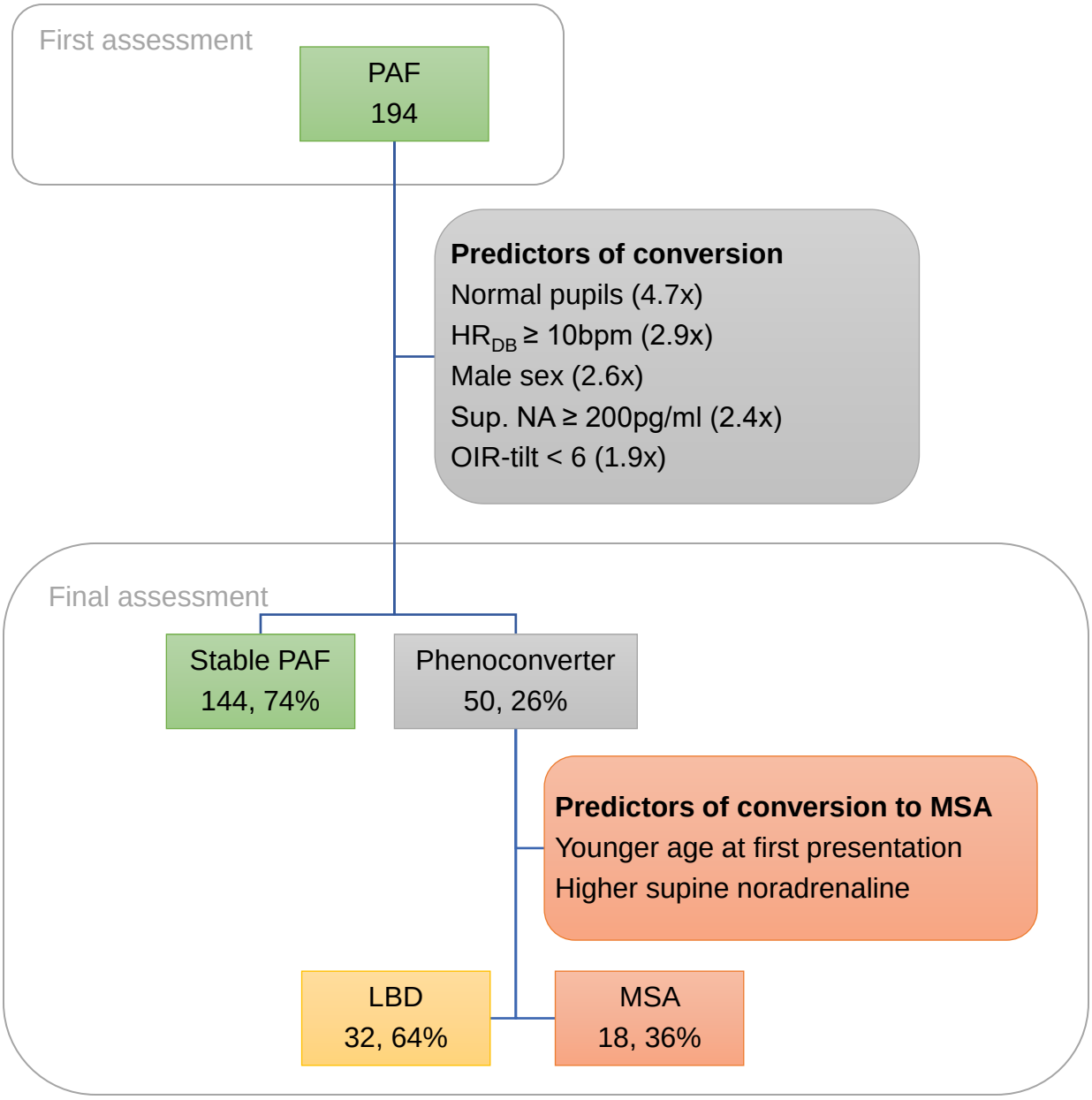


Figure 5.2. Predictors of conversion from PAF to MSA or LBD by the time of final assessment

Table 5.5. Predictors of phenoconversion on univariate logistic regression modelling

Predictors of phenoconversion in patients with PAF	OR, 95% CI	*P*-value
Normal pupils	4.7, 1.4-15.9	.01
HR_{DB} ≥ 10bpm	2.9, 1.5-5.4	.001
Male sex	2.6, 1.3-5.2	.008
Supine noradrenaline ≥ 200pg/ml	2.4, 1.5-3.9	.0004
OIR-tilt < 6	1.9, 1.8-3.0	.01
Predictors of conversion to MSA rather than LBD	**OR, 95% CI**	***P*-value**
Age at initial assessment, years	0.88, 0.81-0.97	.01
Supine noradrenaline, pg/ml	1.01, 1.00-1.03	.04

5.3.5 Prospective patient cohort

52 patients with an α-synucleinopathy were recruited to our prospective natural history study between July 2018 to June 2021, including 28 patients with PAF, 18 with MSA, and 6 with LBD (5 PD, 1 DLB) at recruitment. Patients underwent detailed multi-modal autonomic testing and autonomic symptom and quality of life questionnaires.

At the time of recruitment to the prospective study, patients with PAF had a longer disease duration (median 9, IQR 6-14 years) compared to patients with MSA, median 6 (IQR 3-9) years, *P*=.04 (Table 5.6).

Patients with PAF reported the most severe orthostatic intolerance, with significantly higher orthostatic intolerance subscores than patients with MSA on the COMPASS-31 (median 32 [IQR 28-36] vs 20 [9-28], *P*=.01) and SFN-SIQ questionnaires (3 [2-3] vs 1 [1-2], *P*=.04). On the SF-36 quality of life questionnaire, most patients reported severe impairment of physical function (median 25, IQR 15-49, where 0 indicates severe impairment and 100 indicates no impairment) and role limitations due to physical health

(median 0, IQR 0-25, where 0 indicates severe limitations and 100 indicates no limitations), with no difference between groups.

Table 5.6. Demographics, autonomic symptoms, and quality of life in prospective cohort

	Median, IQR			*P*-value			
	PAF n=28	MSA n=18	LBD n=6	ANOVA	PAF vs MSA	PAF vs LBD	MSA vs LBD
Male, n, %	17, 68	12, 66	5, 83	.83			
Age, y	61, 57-69	63, 54-67	71, 66-73	.24			
Disease dur, y	9, 6-14	6, 3-9	6, 5-10	.047	.04	1	1
COMPASS-31, n	24	14	2				
Total	48, 42-60	37, 29-47	20, 19-21	.01	.10	.06	.57
Orthostatic intol.	32, 28-36	20, 9-28	10, 5-15	.002	.01	.06	1
Vasomotor	0, 0-0	0, 0-0	0, 0-0	.77			
Secretomotor	4.3, 0-8.6	4.3, 0-6.4	2.1, 1.1-3.2	.76			
Gastrointestinal	7.6, 5.1-12.5	8.0, 5.8-10.5	5.8, 3.8-7.8	.77			
Bladder	3.3, 0.8-5.6	3.9, 2.2-6.4	0.6, 0.3-0.8	.10			
Pupillomotor	2, 1.0-2.4	2.3, 0.2-3.0	1.2, 0.6-1.8	.75			
SFN-SIQ, n	25	12	3				
Total	16, 9-18	10, 8-10	12, 8-16	.04	.03	.71	.75
Sweating abnormalities	3, 1-3	0, 0-2	3, 1.5-3	.05	.04	1	.61
Diarrhoea	1, 0-1	0.5, 0-1	0, 0-0.5	.33			
Constipation	1, 1-2	1, 1-2.3	1, 0.5-2	.70			
Micturition problems	2, 1-3	2, 1.8-3	1, 0.5-2	.57			
Dry eyes	0, 0-1	0, 0-0	0, 0-1.5	.15			
Dry mouth	1, 0-3	0, 0-1.3	2, 1-2.5	.16			
Orthostatic dizziness	3, 2-3	1, 1-2.3	3, 1.5-3	.04	.04	1	.88
Palpitations	1, 0-2	0, 0-0.3	0, 0-0	.05			
Hot flashes	0, 0-1	0, 0-0	0, 0-1	.41			
Sensitive skin	0, 0-1	0, 0-0	0, 0-0	.05			
Burning feet	0, 0-0	0, 0-0	0, 0-0.5	.90			
Bedsheet intolerance legs	0, 0-.8	0, 0-0	0, 0-0	.40			
Restless legs	0, 0-2	0, 0-1	1, 0.5-1.5	.80			
SF-36, n	25	15	2				
Physical function	25, 20-50	15, 5-30	45, 25-65	.21			
Role limitations, physical	0, 0-25	0, 0-25	50, 25-75	.71			
Role limitations, emotional	67, 33-100	67, 33-100	50, 25-75	.83			
Energy/fatigue	35, 25-45	40, 30-50	33, 29-36	.55			
Emotional well-being	48, 40-76	36, 32-60	76, 70-82	.18			
Social functioning	50, 38-63	38, 13-63	75, 69-81	.08			
Pain	58, 33-90	70, 41-90	61, 47-76	.93			
General health	50, 30-65	50, 23-58	28, 26-29	.36			

5.3.6 Cardiovascular autonomic testing and plasma catecholamines in prospective cohort

Cardiovascular testing and plasma catecholamines at recruitment to the prospective study showed similarities to the findings from the retrospective study (Table 5.7). Patients with PAF demonstrated more severe orthostatic hypotension. Systolic blood pressure on standing fell markedly across all groups, but significantly more in patients with PAF (median ΔSBP 81 [62-96] mmHg) compared to patients with MSA (40 [30-77] mmHg, *P*=.03). OIR-stand (ΔSBP over time tolerated standing, up to 5 minutes) was significantly higher in patients with PAF compared to MSA (18 [13-25] vs 8 [6-17], *P*=.03). OIR-tilt was also highest in the PAF group (11 [7-27]) compared to the MSA group (6 [4-11), although this was not significant at the 5% level (*P*=.06).

PRT after the Valsalva manoeuvre was most prolonged in the PAF group (median 23 [IQR 19-29] seconds compared to 17 [6-26] seconds in the MSA and 12 [10-16] seconds in the LBD groups, although this was not significant at the 5% level *P*=.07). Supine noradrenaline was significantly lower in patients with PAF (167 [140-190] pg/ml) compared to patients with MSA (246 [234-303] pg/ml, *P*<.001), with intermediate levels seen in patients with LBD (216 [158-223] pg/ml). The rise in noradrenaline with tilt was lowest in patients with PAF (4 [1-13] pg/ml) and highest in patients with LBD (38, 23-67 pg/ml, *P*=.02).

5.3.7 DST

48 patients had DST performed at the distal leg (26 PAF, 17 MSA and 4 LBD). All groups had abnormal postganglionic sudomotor dysfunction, with reduced sweat output (76 [53-158] nL/cm^3/min, normal ≥ 417 nL/cm^3/min), sweat gland density (38 [25-53] sweat

glands/cm^3, normal ≥ 64 glands/cm^3), and sweat output/gland (2.5 [1.3-3.8] nL/min, normal ≥ 5.6 nL/min), with no differences between groups.

Table 5.713. Cardiovascular autonomic testing and plasma catecholamines in prospective cohort

	Median, IQR			*P*-value			
	PAF, n=28	MSA, n=18	LBD, n=6	ANOVA	PAF vs MSA	PAF vs LBD	MSA vs LBD
Supine							
SBP, mmHg	151, 142-170	133, 118-152	161, 131-188	.03	.03	1	.42
HR, bpm	66, 58-73	70, 61-76	67, 65-67	.66			
Stand							
Δ SBP, mmHg	81, 62-95	40, 30-77	80, 49-102	.03	.03	.88	.40
Δ HR, bpm	7, 3-16	11, 4-17	17, 13-20	.44			
OIR-stand	18, 13-25	8, 6-17	16, 10-21	.03	.03	.65	1
Tilt							
Δ SBP, mmHg	76, 57-97	62, 40-78	74, 41-105	.32			
Δ HR, bpm	1, -7 to 18	7, 0-14	13, -3 to 23	.64			
OIR-tilt	11, 7-27	6, 4-11	8, 4-17	.06			
Isometric exercise							
Δ SBP, mmHg	0.5, -5 to 0	4, -2 to 9	5, -2 to 17	.40			
Δ HR, bpm	3, 1-6	5, 3-7	10, 6-14	.07			
PRT, s	23, 17-29	17, 6-26	13, 10-16	.07			
Valsalva ratio	1.22, 1.11-1.37	1.20, 1.13-1.34	1.14, 1.10-1.22	.77			
HR_{DB}	4.5, 0-8	4, 0.5-7	4, 4-6	.98			
Noradrenaline, n	28	16	5				
Supine, pg/ml	167, 140-190	246, 234-303	216, 158-223	2 e-8	<.001	.19	.02
Δ NA-tilt, pg/ml	4, 1-13	11, 5-13	38, 28-67	.02	.53	.02	.32
Dynamic sweat testing, distal leg, n	26	17	5				
Sweat output, nL/cm^3/min	75, 45-137	72, 55-155	107, 96-166	.47			
Sweat glands/cm^3	38, 27-53	37, 20-51	47, 34-53	.62			
Sweat output/gland, nL/cm^3/min [2]	2.1, 1.2-3.3	2.5, 1.3-4.3	2.9, 2.9-3.8	.61			

SBP, systolic blood pressure; HR, heart rate; NA, noradrenaline.

5.3.8 Bladder studies

A third of MSA patients (6/18) used urinary catheters at their first assessment and a further third (6/18) were subsequently referred for CISC due to persistently elevated PVR (67%, 12/18 in total). Fewer of the PAF patient used urinary catheters at first assessment (14%, 4/28), with a further 21% (6/28) patients subsequently referred for CISC (35%, 10/28 in total, *P*=.04)

35 patients had documentation of PVR (13 patients with MSA, 22 with PAF) and 32 had uroflowmetry performed (10 with MSA, 22 with PAF). Of the 13 MSA patients with PVR recorded, 11 patients (85%) had elevated PVR >100ml, compared to 7/22 (32%) of PAF patients (*P*=.02).

Of the 10 MSA patients with uroflowmetry performed, one patient with overactive bladder symptoms voided a small volume <100ml so the flow profile could not be analysed. Flow profile was abnormal in 7/9 of the patients who voided at least 100ml (78%). The two patients with normal flow profiles had PVR of 122-156ml, meaning none of the MSA patients had a normal bladder assessment.

Of the 22 PAF patients with uroflowmetry performed, 3 patients voided <100ml, meaning flow pattern could not be assessed. Two of these patients were already known to have urinary retention and used CISC. Of the 19 patients voiding at least 100ml, 13 (68%) had abnormal flow profiles. Amongst the patients with normal flow profiles, 1 had a mildly elevated PVR of 121ml and the other 5 had PVR <100ml.

Table 5.814. Bladder studies in prospective cohort

	PAF, n=28	MSA, n=18	*P*-value
Urinary catheters, n, %			
At first assessment	4, 14	6, 33	.16
During study	10, 35	12, 67	.04
Post-void residual volume, n	22	13	
Post-void residual volume, ml	52, 3-131	173, 142-250	.01
Post-void residual volume >100ml, n, %	7, 32	10, 83	.02
Uroflow performed, n	22	10	
Volume voided >100ml, n	19	9	
Uroflow abnormal, n, %	13, 68	7, 78	.68

5.4 Discussion

This is the largest study evaluating multiple objective autonomic biomarkers in patients with α-synucleinopathies seen at a single autonomic unit over 20 years, revealing important differences in the autonomic phenotype of patients with PAF, MSA and LBD, and autonomic biomarkers that can predict phenoconversion in patients presenting with PAF. On multimodal autonomic testing, patients with PAF demonstrated more severe orthostatic hypotension, lower supine noradrenaline levels, and more frequent sympathetic pupillary deficits compared to patients with MSA, in keeping with greater post-ganglionic adrenergic dysfunction. Compared to patients with MSA, patients with PAF reported more severe orthostatic intolerance, but similarly severe levels of impairment in daily activities and role limitations due to physical health, suggesting significant physical disability despite an absence of motor symptoms, likely due to their symptomatic orthostatic hypotension.

5.4.2 Severe symptomatic orthostatic hypotension and postganglionic adrenergic failure in PAF

In our retrospective study, the patients with PAF had the most severe orthostatic hypotension at first assessment, with highest ΔSBP with head-up tilt and OIR-tilt, as well as lowest ΔHR with isometric exercise, Valsalva ratio, and supine plasma noradrenaline levels, with the majority demonstrating sympathetic pupillary deficits, in keeping with more severe postganglionic adrenergic denervation. In contrast, the patients with MSA typically had higher supine heart rates, with preserved supine noradrenaline levels and pupillary function, consistent with intact postganglionic adrenergic innervation.[106] These findings are consistent the study of Mabuchi et al, that showed patients with PAF had more severe ΔSBP on tilt, lower supine noradrenaline and lower H:M ratio on cardiac MIBG indicating greater postganglionic adrenergic denervation compared to patients with MSA.[51]

Our prospective study showed patients with PAF consistently significantly more orthostatic intolerance symptoms on the COMPASS-31 and SFN-SIQ, that in keeping with more severe orthostatic hypotension on contemporaneous objective autonomic testing. They reported similar impairments of physical activities due to health problems and role limitations due to their physical health compared to patients with MSA, despite a lack of motor symptoms. Our study highlights the fact that severe symptomatic orthostatic hypotension, even in the absence of other neurological symptoms and signs, can be associated with significant disability, and deserves prompt recognition, assessment, and treatment.

5.4.3 Urinary studies in PAF and MSA

In our study, a significantly higher proportion of patients with MSA had elevated PVR >100ml and required catheterisation compared to patients with PAF, consistent with previous studies describing severe, early urinary dysfunction in patients with MSA and occurring as a late feature patients with PAF.[51] Nevertheless, it is worth noting that almost a third of patients with PAF had urinary retention at first assessment, and of the patients with PVR <100ml, 9/14 (64%) had an abnormal flow pattern, of whom two went on to develop persistently elevated PVR requiring catheterisation. Our data suggests that urinary dysfunction is not an uncommon feature in patients with PAF, who should all have a basic bladder assessment, ideally with uroflowmetry and PVR. Patients with persistently elevated PVR >100ml should be referred for CISC training, and patients with abnormal uroflowmetry without urinary retention at initial assessment should have interval studies to monitor for development of urinary retention.

5.4.4 Phenoconversion from PAF to MSA/LBD

In keeping with previous natural history studies, 26% of the patients initially diagnosed with PAF in our retrospective study converted to either MSA or LBD by the end of the study perioed.[15-18] Having normal pupils, more preserved heart rate variability with deep breathing, supine noradrenaline and less severe orthostatic intolerance on tilt, as measured by the OIR-tilt on initial assessment were associated with significantly increased odds of future phenoconversion to MSA or LBD. Younger age and higher supine noradrenaline at first assessment predicted conversion to MSA rather than LBD. The results are in agreement with previous natural history studies which found patients with PAF who later converted to MSA/LBD had less severe ΔSBP on tilt on initial

assessment,[16] and patients converting to MSA rather than LBD were younger at first presentation,[18] with higher supine noradrenaline.[15, 17, 18] Our study is the first to systematically evaluate pupillary function in a large cohort of patients with autonomic failure with longitudinal follow up and suggests it is a novel non-invasive biomarker that can help predict future phenoconversion. We would advocate including pupillometry in the work up of all patients presenting with autonomic failure. The validation of our current pupillometry protocol with handheld pupillometers would enable bedside pupillometry to be performed in patients with more physical disability who are unable to tolerate an additional visit to the neuro-ophthalmology clinic either due to severe motor symptoms or severe orthostatic intolerance.

5.4.5 Limitations

We evaluated a large cohort of patients with rare neurodegenerative diseases with a consistent protocol of autonomic testing over 20 years in our retrospective study, and used a comprehensive multimodal assessment and patient reported outcomes for patients recruited to our prospective study. The patients we studied were all referrals to a national autonomic unit, who are more likely to have severe autonomic symptoms due to referral bias. In particular, the patients we are referred with PD tend to be those with prominent autonomic features or other atypical features, meaning our findings are not representative of the more typical patient with idiopathic Parkinson's disease.

5.4.6 Future directions

In summary, our study showed differences in the cardiovascular, neurohormonal, pupillary and urinary profiles of patients with α-synucleinopathies, with features consistent with postganglionic adrenergic dysfunction commonly seen in patients with

PAF. Relatively preserved cardiovascular autonomic function, supine noradrenaline and pupils in patients presenting with PAF predicted phenoconversion to a more widespread α-synucleinopathy. Severe symptomatic orthostatic hypotension is likely to have a major impact on the physical function in patients with PAF even in the absence of other neurological features and merits prompt assessment and treatment.

Chapter 6. Cutaneous p-syn differentiates PAF from other α-synucleinopathies and non-synucleinopathy related diseases

6.1. Introduction

6.1.1 Diagnostic challenges in early presentations of isolated autonomic failure

Patients presenting with isolated autonomic failure in the absence of other neurological deficits represent a diagnostic challenge. Clinical features such as a subacute or post-infectious onset of severe pandysautonomia, with plateauing of disease progression, spontaneous remission, or improvement following immune therapy, the presence of other autoimmune or neoplastic disease, may suggest an autoimmune aetiology, even in the absence of known antibodies associated with autoimmune autonomic failure, whereas a more gradual onset and relentlessly progressive autonomic failure in a more elderly patient, with more subtle motor findings that are too mild to definitely discern from normal variants or normal aging,[17] or symptoms suggestive of RBD, may point towards an underlying α-synucleinopathy as the cause of the symptoms. However, at the early stages of presentation, the overall trajectory of the disease and other diagnostic clues may not yet be evident, and individual patients may present with atypical features that lead to diagnostic challenges.

In patients who are referred several years after symptom onset, it can be difficult to clearly recall the nature and circumstances around the onset of symptoms. Patients with an autoimmune aetiology that is not recognised early stage may report progressive worsening of their symptoms with ongoing untreated disease and aging.[1, 72] In contrast, patients with a neurodegenerative disease which is more gradually progressive disease

may adapt to slowly worsening physiological abnormalities so they are barely aware of them, but decompensate following an intercurrent infection or operation, and suddenly become aware of symptoms, reporting a more acute/subacute onset that appears to be triggered by an infection or procedure. Segmental or regional anhidrosis may suggest a patchy, ganglionic pathology, in keeping with an autoimmune aetiology such as Sjogren's disease[94] or autoimmune autonomic ganglionopathy,[70] but there have been reports of patients presenting initially with unilateral flushing and sweating and contralateral anhidrosis, in keeping with Harlequin's syndrome, later developing progressive orthostatic intolerance and cardiovascular autonomic failure in keeping with pure autonomic failure.[107, 108] In the absence of detectable antibodies associated with autoimmune autonomic failure and other clinical features or biomarkers to confirm a specific diagnosis, clinicians may offer patients an empirical trial of immune therapy for possible autoimmune disease.

6.1.2 Cutaneous α-synuclein as a diagnostic biomarker for PAF

Cutaneous synuclein is a potential non-invasive diagnostic biomarker to confirm an underlying synucleinopathy in patients presenting with isolated autonomic failure. Donadio et al previously studied 21 patients with chronic peripheral autonomic neuropathy, including 12 patients who had an acquired autonomic neuropathy including patients with diabetes, hepatitis C and autoimmune connective tissue disease, and 9 patients without a definite cause for their autonomic failure and no other neurological involvement, fulfilling diagnostic criteria for PAF.[43] All PAF patients showed α-synuclein deposits, and none of the acquired autonomic neuropathy patients did. The group later showed their cohort of patients with PAF had more abundant and homogenous synuclein

deposition compared to patients with idiopathic PD.[44] Isonaka et al found higher indices of α-synuclein colocalization with sympathetic neurons in patients with Lewy body pathology, defined as PAF, DLB, and PD, compared to patients with non-Lewy body disease, including MSA and other non-synuclein related pathology, both in skin biopsies and sympathetic ganglia in patients who had post-mortem studies.[109] α-synuclein colocalization indices correlated with reduced in vivo cardiac dopamine uptake studies and myocardial noradrenaline on post-mortem studies, suggesting intraneuronal synuclein deposition may lead to myocardial sympathetic neurodegeneration in Lewy body pathology. However, no studies to date have explored the relationship between cutaneous synuclein deposition and autonomic innervation with quantitative markers of cardiovascular autonomic failure.

6.1.3 Aims of study

I prospectively studied patients referred to a national autonomic centre with autonomic failure with α-synucleinopathies (including PAF, MSA and PD) and other non-synucleinopathy related diseases with multimodal autonomic testing and skin biopsies to analyse the presence and distribution of cutaneous p-syn, to establish whether this might be able to distinguish between:

1) PAF and non-synucleinopathy diseases affecting the autonomic nervous system
2) PAF and other α-synucleinopathies, including MSA and PD.

Secondly, I studied the relationship between cutaneous p-syn, somatic and autonomic innervation and quantitative markers of cardiovascular autonomic failure to try gain insight into pathogenesis of autonomic failure in α-synucleinopathies.

6.2 Methods

103 patients were recruited prospectively for a natural history study from April 2018 to June 2021 and underwent detailed multimodal autonomic testing including cardiovascular autonomic testing, plasma catecholamines, pupillometry, bladder assessment, dynamic sweat testing, punch skin biopsies to quantify intraepidermal and pilomotor nerve fibre density and assess for the presence of α-synuclein, and standardised autonomic symptom questionnaires. They were followed up until December 2022.

39 patients had punch skin biopsies analysed for intraepidermal and pilomotor innervation and cutaneous phosphorylated synuclein, including 27 patients with α-synucleinopathies (11 with PAF, 13 with MSA, 3 with PD). Skin biopsies from 12 other patients with non-synuclein related pathology were also studied, including 8 with autoimmune autonomic failure (6 gAChR-positive, 2 gAChR-negative), 2 with progressive supranuclear palsy, 1 with hereditary transthyretin amyloidosis, and 1 with chemotherapy induced anhidrosis.

6.2.1 Cardiovascular autonomic testing and plasma catecholamines

All patients underwent cardiovascular autonomic testing as previously described[55] with beat-to-beat recordings of blood pressure and heart rate at rest in the resting supine position and with:

1) Passive head up tilt to 60° for up to 10 minutes
2) Isometric exercise (sustained handgrip for 3 minutes at a third of maximum voluntary contraction pressure)
3) Deep breathing, at a rate of 6 breaths/min

4) Valsalva Manoeuvre (forced expiration at 40mmHg for 10 seconds)

Patients underwent an active stand for up to 5 minutes and were passively tilted for a maximum of 10 minutes. Some patients developed severe orthostatic hypotension with signs of cerebral hypoperfusion on orthostasis necessitating early termination of tilt. OIR-tilt was calculated by dividing the ΔSBP in mmHg over the time tolerated on head-up tilt in minutes, up to a maximum of 10 minutes.

Valsalva ratio was calculated by dividing maximum heart rate developing during Phase II of the Valsalva manoeuvre over the minimum heart rate occurring within 30 seconds of the peak heart rate. PRT was defined as the time taken for the systolic blood pressure to recover from Phase III back to the baseline.[56]

Blood samples were collected via intravenous forearm catheter at rest and following orthostasis for analysis of plasma noradrenaline using high performance liquid chromatography.

6.2.2 Sudomotor testing

Patients underwent DST, an assessment of postganglionic sudomotor function, at the distal leg and forearm bilaterally.[63] After iontophoresis with 1% pilocarpine, skin was coated with iodine and formation of sweat gland imprints on starch covered tape was recorded. Density of activated sweat glands/cm^3, sweat output/min/cm^3, and average sweat output/gland was calculated for each site and the mean value for both sides used. A length dependent deficit was defined as sweat output at the distal leg site less than the 5^{th} percentile of normal values and less than 1/3 of sweat output at the forearm.[90]

6.2.3 Morphological analysis of punch skin biopsies

All patients had 3mm punch skin biopsies collected under local anaesthetic from the distal leg bilaterally, and some patients had additional samples collected from the anterior thigh and forearm. To maximise the sampling of arrector pili muscles and sweat glands, biopsies were centred around a hair follicle. Samples were placed into chilled Zamboni solution and then transferred to cryoprotectant solution after 4-6 hours. Samples were sliced into 50µm thick sections using a freezing sliding microtome (Leica 2000) and processed for indirect immunofluorescence according to standard procedures using a large panel of antibodies, including primary antibodies against ColIV, PGP, a pan-neuronal marker, VIP, a cholinergic marker, DβH, an adrenergic marker, and p-syn, and species-specific secondary antibodies coupled with Cy2, Cy3 and Cy5 fluorophores.[91] Digital confocal images were acquired using a non-laser confocal system (Apotome2 Zeiss, Jena, Germany, EU).

6.2.3.1 Quantification of intraepidermal and pilomotor fibres

IENF density was measured as the number of fibres crossing the dermal-epidermal junction according to current guidelines.[69] Quantification of pilomotor fibres was performed as previously described.[39] Briefly, arrector pili muscle segments parallel to the focal plane were acquired using a 20x objective. The single optical section with the most fibres running for at least 100 µm parallel to the major axis of the muscle was selected from the Z-stack. A line was then traced perpendicular to the major axis intercepting the most fibres. Pilomotor nerve fibre density was calculated as the average number of intercepts per muscle width in fibres/mm of all muscles suitable for quantification for each staining for each biopsy.

6.2.3.2 Semiquantitative assessment of cutaneous neural p-synuclein deposition

Sections double stained with pan-neuronal marker PGP and p-syn were studied to search for the presence of p-syn in all axons around cutaneous autonomic adnexa, including the 1) sweat glands, 2) blood vessels, and 3) arrector pili muscles, as well as 4) subepidermal fibres, 5) dermal nerve fibres, and 6) nerve fascicles. P-syn was considered present if it was seen in at least one of each of the six structures listed above. An autonomic p-syn score out of 3 and total p-syn score out of 6 was noted for each biopsy (Figure 6.1).

When bilateral samples were available, the mean cutaneous intraepidermal and pilomotor nerve fibre density, total p-syn score and autonomic p-syn subscore of the two sides was taken as representative for that patient.

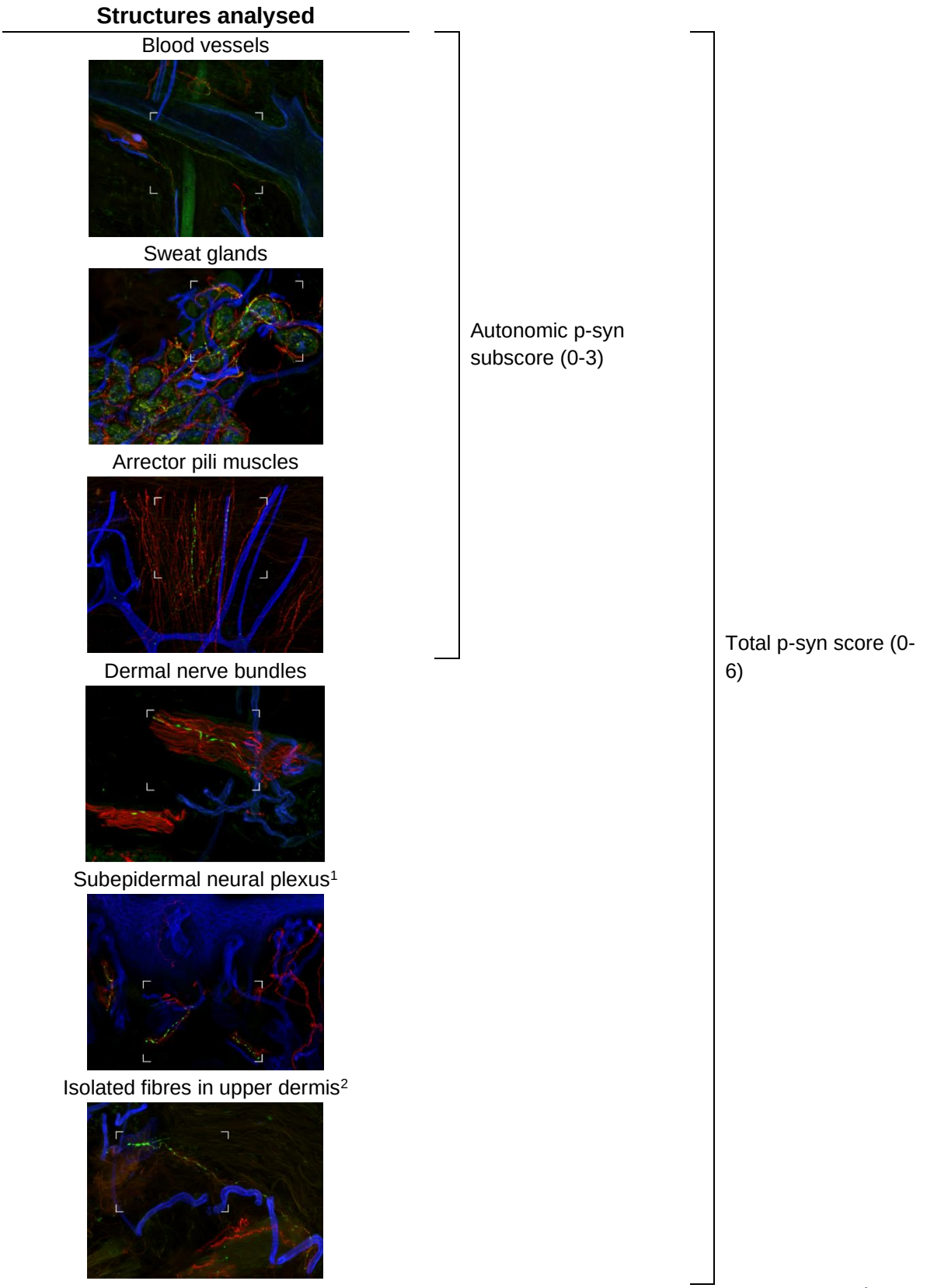


Figure 6.1. Summary of structures used for calculating total p-syn score and autonomic p-syn subscore. [1]<200µm below basement membrane,[2]200- 500µm below basement membrane

6.2.4 COMPASS-31

Autonomic symptoms at recruitment were recorded using the abbreviated and refined composite autonomic symptom score (COMPASS-31). The COMPASS-31 assesses patient symptoms in six autonomic domains, with weighted subscores for orthostatic intolerance, vasomotor, secretomotor, gastrointestinal, bladder, and pupillomotor symptoms, giving an overall autonomic symptom score from 0 to 100.[66]

6.2.5 Statistical analysis

Data were captured electronically using a secure Research Electronic Data Capture (REDCap) platform. Statistical analysis was performed using R Studio, Version 1.2.1335. Summary data has been displayed as median, interquartile range for continuous data and numbers, percentages for categorical data. Distributions of data were assessed for normality by visual inspection and using Shapiro-Wilk tests. Pairwise comparisons were made with unpaired two-tailed T-tests/Wilcoxon rank-sum tests and group comparisons were made with ANOVA/Kruskal-Wallis tests with post-hoc comparisons using Tukey/Dunn's tests with Bonferroni corrections as appropriate. Chi-squared tests were used to compare categorical data. Spearman's rank correlation was used to assess the correlation between linear variables. $P<.05$ was considered significant.

6.3. Results

6.3.1 Cutaneous p-syn in PAF and non-synucleinopathy related diseases

Skin biopsy samples from 11 patients with PAF were compared to 12 patients with non-synucleinopathy related diseases were analysed for the presence of p-syn deposits on cutaneous nerves.

P-syn deposits were visualised in all 26/26 samples from the PAF patients, and none of the 31 samples from the non-synucleinopathy patients. Mean intraepidermal and pilomotor nerve fibre density at the distal leg at were reduced in both PAF and non-synucleinopathy groups compared to normal cut off values for age and sex, with no significant difference between groups (Table 6.1)

Table 6.1. Phosphorylated synuclein in PAF and non-synucleinopathy patients

	PAF	Non-synucleinopathy
Patients studied, n	11	12
Age at biopsy, years	57, 55-65	54, 43-61
Female, n, %	3, 27%	6, 50%
P-syn positive, n, %	11, 100%	0, 0%
Total samples, n	26	31
P-syn positive, n, %	26, 100%	0, 0%
Forearm	NA	0/2, 0%
Thigh	4/4, 100%	0/9, 0%
Distal leg	22/22, 100%	0/22, 0%
Cutaneous innervation, distal leg		
IENF, f/mm	1.8, 0.9-2.8	1.5, 0.5-6.0
Pilomotor nerve fibre density, f/mm	22, 11-34	19, 15-29

When reviewing sections co-stained with PGP and p-syn, most samples from patients with PAF demonstrated abundant neural p-synuclein deposits. Arrector pili muscles were visualised in sections from 23 samples and sweat glands in 20 samples. P-syn deposits were frequently visualised on pilomotor nerve fibres (96%, 22/23 samples) , nerve fibres within dermal nerve bundles (93%, 24/26 samples) (Figure 6.2), sudomotor fibres (80%, 16/20 samples) and nerves running alongside blood vessels (69%, 18/26 samples). P-syn deposits were less commonly seen on fibres in the subepidermal plexuses (46%, 12/26 samples) and isolated nerve fibres running deeper within the dermis (35%, 9/26 samples).

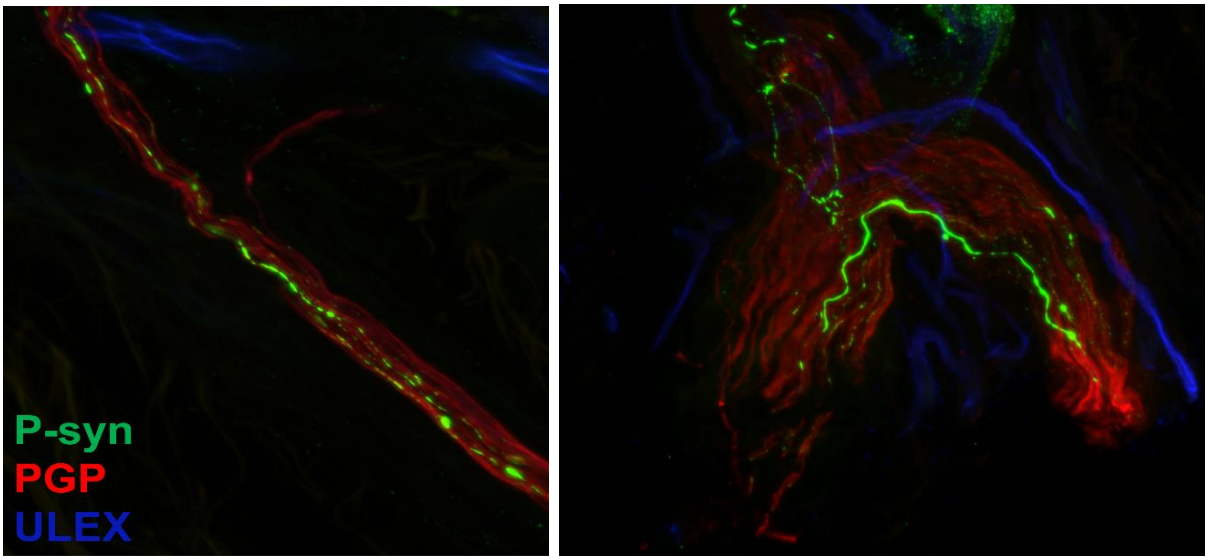


Figure 6.6. Confocal images of cutaneous p-syn deposits from a patient with PAF. P-syn is seen to co-localise along nerve fibres, marked with PGP, running within dermal nerve bundles.

6.3.1.1 Cutaneous p-syn as a diagnostic biomarker in PAF patients with atypical features

Amongst the 11 PAF patients studied, 4 patients had atypical features at initial presentation, leading to trials of immune therapy by their treating clinicians for possible gAChR-negative autoimmune autonomic failure, either prior to referral, for the first

patient, or at our centre, in the last three patients, with no objective clinical benefit, before developing progressive features that led to a revision of their diagnoses to probable PAF.

In contrast to the majority of patients with PAF seen at our centre in the last 20 years, described in Chapter 5, who were older at first evaluation, with a median age of 68 years (IQR 59-75 years), presenting with gradually progressive orthostatic intolerance over several years with generalised anhidrosis, these 4 patients presented at a younger age, between 49-55 years, with atypical and severely disabling symptoms, which led to initial diagnostic uncertainty, summarised in Box 6.1.

All 4 patients had clear evidence of p-syn on skin biopsies collected on recruitment to our natural history study, which were collected prior to the development of RBD and any other central features in 3 of the 4 patients. The use of minimally invasive skin biopsies to confirm a neurodegenerative alpha-synucleinopathy as the cause of their autonomic failure at an early stage of their disease would have averted the trials of immune therapy and associated complications and costs of treatment.

Box 6.1 Patients with atypical features who received trials of immunotherapy

1. A 50-year-old man developed severe orthostatic intolerance, erectile dysfunction, bloating and vomiting. Prominent upper gastrointestinal symptoms suggestive of cholinergic autonomic failure are common in patients with autoimmune pathology and very unusual in PAF. A trial of IVIg had no effect. When assessed at our unit, 4 years after onset, his symptoms had continued to progress. He had anosmia, with subtle motor signs not fulfilling criteria for parkinsonism, consistent with a likely α-synucleinopathy.

2. A 44-year-old man developed progressive orthostatic intolerance, anhidrosis, diarrhoea, and urinary retention requiring intermittent catheterisation, with severe widespread autonomic failure on testing 5 years after onset. He had 2 courses of plasma exchange with no benefit. After 2 years, he developed symptoms consistent with RBD, mild rigidity and bradykinesia, with an abnormal DATscan and MIBG, suggesting evolving PD with autonomic failure.

3. A 55-year-old man was referred with an 8-year history of erectile dysfunction, followed by left sided anhidrosis over the head, torso and arm, with compensatory right sided hyperhidrosis (Figure 6.3), and orthostatic intolerance. 3 courses of plasma exchange had no benefit and he continued to progress. 11 years after onset, he developed symptoms suggestive of RBD.

4. A 55-year-old man presented with a 12-year history of erectile dysfunction, hypohidrosis, severe constipation, initially mild orthostatic intolerance that eventually became severely debilitating, and urinary retention requiring catheterisation. He had 12 courses of plasma exchange over 2 years, reporting symptomatic benefit with initial exchanges, but no consistent improvements on objective testing, unfortunately developing a deep vein thrombosis requiring anticoagulation. With oral prednisolone 60mg, he had improved orthostatic tolerance but with significant supine hypertension and developed bilateral cataracts. 15 years after onset, he developed violent dream enactment behaviour, with RBD confirmed on polysomnography.

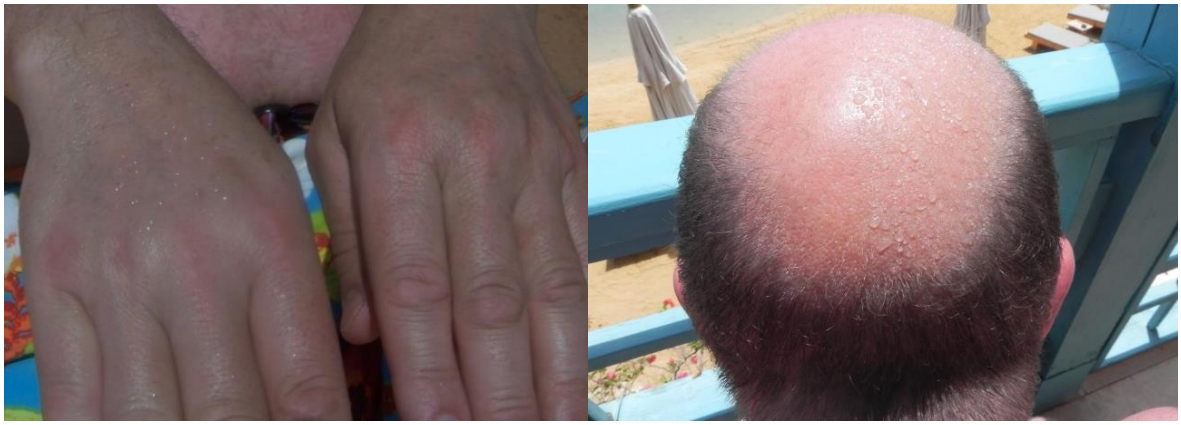


Figure 6.3. Left-sided anhidrosis with right-sided hyperhidrosis in Case 3, pictures supplied by patient.

6.3.2 Differences in total and autonomic p-syn scores between PAF and other α-synucleinopathies

The clinical characteristics and autonomic phenotype of patients with α-synucleinopathies recruited to our prospective study were described fully in Chapter 5. The findings in the subset of patients with biopsies are summarised in Table 6.2. Bilateral distal leg skin biopsies were available in 27 patients with α-synucleinopathies including 11 with PAF, 13 with MSA, 3 with PD.

Intraepidermal and pilomotor innervation with pan-neuronal marker PGP were reduced in all synucleinopathy patients compared to normal values, with no difference between groups (median IENF 1.8, IQR 1-3.7 f/mm; median pilomotor nerve fibre density 29, 19-37 f/mm). Cutaneous p-syn was present in 24/27 synucleinopathy patients: 11/11 with PAF, 12/13 with MSA, and 1/3 with PD, versus 0/12 of non-synuclein controls (Figure 6.4).

The samples from patients with PAF had abundant p-syn deposition in comparison to the other α-synucleinopathies. The total p-syn score was significantly higher in patients with PAF (median 4.5 [IQR 3.8-4.8]) compared to patients with MSA (1 [0.5-1.5], *P*<.001) and PD (0 [0-0.8], *P*=.01). Autonomic p-syn subscore, reflecting synuclein deposition in nerves supplying the autonomic adnexa, followed a similar pattern (2.5[1.8-3] in PAF compared to 0.5 [0.5-1] in MSA, *P*<.001, and 0, [0-0.5] in PD, *P*=.01)

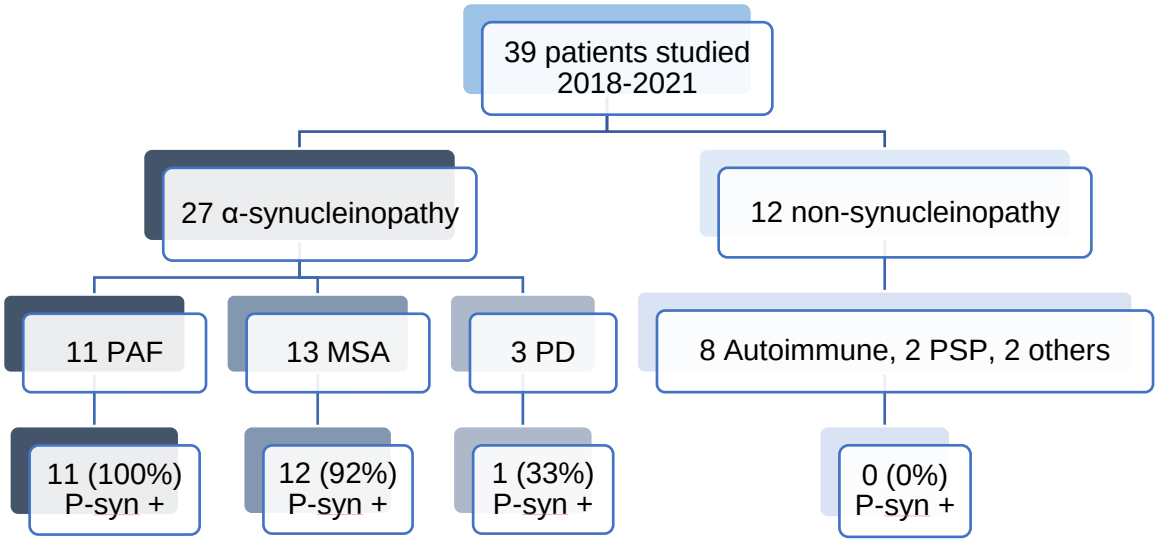


Figure 6.4. P-syn positivity in α-synucleinopathy and non-synucleinopathy patients.

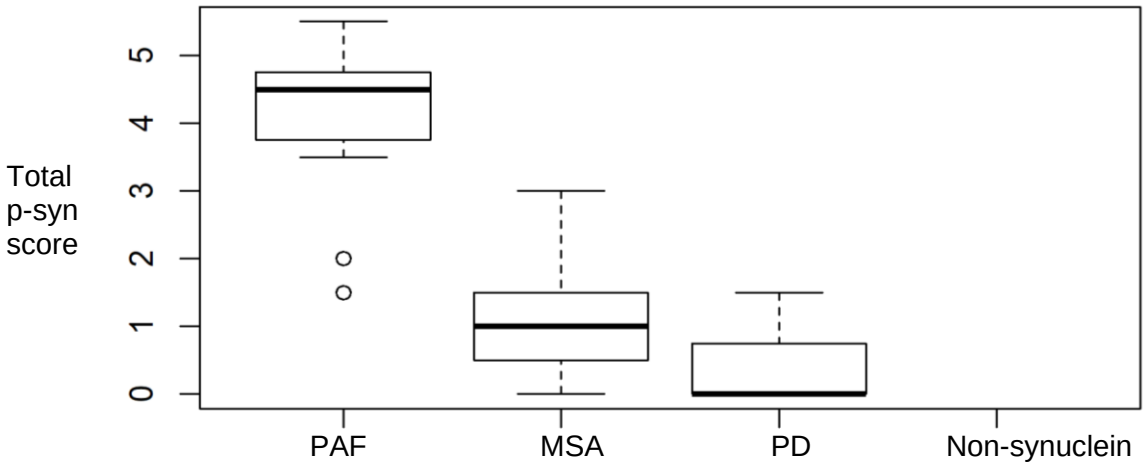


Figure 6.5. Total p-syn score was significantly higher in patients with PAF (median 4.5 [IQR 3.8-4.8]) compared to MSA (median 1 [IQR 0.5-1.5], P<.001) and PD (median 0 [IQR 0-0.8], P<.01). None of the non-synucleinopathy related patients had any p-syn deposits.

Table 6.15. Demographic details, autonomic testing, plasma noradrenaline, skin biopsies, and COMPASS-31 scores at recruitment in patients with α-synucleinopathies.

	Median, IQR			*P*-value			
	PAF, n=11	MSA, n=13	PD, n=3	ANOVA	PAF vs MSA	PAF vs PD	MSA vs PD
Female sex, n, %	4, 37	3, 23	1, 33	.89			
Age, years	59, 56-63	64, 60-68	64, 55-68	.19			
Disease duration, years	9, 6-12	6, 3-8	6, 5-6	.15			
Cardiovascular testing							
Supine							
SBP, mmHg	172, 150-192	134, 123-157	141, 132-176	.23			
HR, bpm	65, 57-72	62, 57-73	67, 67-78	.58			
Head-up tilt							
Time tolerated, min	7, 3-10	10, 10-10	10, 8-10	.09			
Δ SBP, mmHg	97, 62-123	62, 40-82	38, 20-68	.10			
Δ HR, bpm	0, -9 to 3	2, -1 to 12	8, 1-13	.50			
OIR - tilt	19, 12-28	6, 4-10	4, 2-12	.02	.04	.16	1
Isometric exercise							
Δ SBP, mmHg	-2, -13 to 1	4, -3 to 9	16, 10-21	.03			
Δ HR, bpm	3, 1-4	5, 3-6	12, 8-17	.23			
Pressure recovery time, s	23, 18-30	16, 9-24	7, 5-10	.04	.23	.06	.50
Valsalva ratio	1.13, 1.11-1.27	1.19, 1.12-1.36	1.10, 1.07-1.33	.64			
HR_{DB}	3, 0-5	4, 2-7	4, 2-6	.54			
Noradrenaline, pg/ml							
Supine noradrenaline	180, 156-202	240, 303-353	187, 220-223	<.001	<.001	1	.13
Δ NA with tilt	12, 2-19	11, 5-12	67, 53-59	.04	1	.06	.049
DST, distal leg							
Sweat output	74, 41-140	63, 53-155	131, 219-272	.34			
Sweat glands/cm^2	36, 25-41	25, 18041	47, 40-49	.23			
Sweat output/gland	3.0, 1.5-3.4	3.6, 2.0-4.3	3.8, 2.2-4.6	.35			
Skin biopsies, distal leg							
IENF, f/mm	1.8, 0.9-2.8	1.5, 0.8-4.9	4.0, 2.6-4.0	.70			
Pilomotor nerve fibre density, f/mm	22, 11-34	28, 20-44	37, 33-38	.30			
Total p-syn score	4.5, 3.8-4.8	1, 0.5-1.5	0, 0-0.8	<.001	<.001	.01	1
Autonomic p-syn subscore	2.5, 1.8-3	0.5, 0.5-1	0, 0-0.5	<.001	<.001	.01	1
COMPASS-31, n	10	11	1				
Total	55, 43-65	33, 33-48	18	.12			
Orthostatic intolerance	34, 29-36	20, 4-28	0	.01	.03	.15	1

NA, noradrenaline

6.3.4 Low total p-syn scores in patients with PAF: a potential predictor of phenoconversion

Amongst the patients recruited with PAF, 2 out of the 3 patients with mean total p-syn scores <4 developed features of a more widespread synucleinopathy by December 2022, suggesting that a low mean p-syn score may be a potential predictor for phenoconversion. These cases are described further in Box 6.2.

Box 6.2 Patients with low p-syn scores who phenoconverted to LBD and MSA

1. The PAF patient with the lowest mean total p-syn score at recruitment (1.5) was a 68-year-old woman with a 7-year history of orthostatic intolerance, hypohydrosis, urinary frequency, diarrhoea, hyposmia, and RBD. She subsequently developed progressive cognitive difficulties, describing visual misperceptions and disorientation in unfamiliar surroundings, with MRI brain demonstrating moderate brain volume loss without regional predilection. Formal neuropsychometry 1.5 years after recruitment demonstrated significant intellectual under-functioning, reduced processing speed and executive dysfunction, with particularly poor visual-spatial function, in keeping with evolving Dementia with Lewy Bodies.

2. A 55-year-old man was recruited with 5-year history of orthostatic intolerance, urinary retention requiring catheterisation, bowel and sudomotor symptoms, on a background of longstanding hyposmia and RBD, with a mild intermittent positional tremor in one arm on examination but no definite Parkinsonism. Mean total p-syn score was amongst the lowest of the PAF cohort at recruitment (3.5) and even lower on follow up samples collected after 1 year (1). Three years after recruitment, he developed increasing rigidity, bradykinesia, slowed speech, a stooped, shuffling gait, and falls, with an abnormal DATscan and normal MIBG, in keeping with MSA.

6.3.5 Relationship between autonomic p-syn score, cardiovascular autonomic testing, and patient-reported symptoms

Mean autonomic p-syn score correlated with cardiovascular autonomic markers influenced by control of total peripheral resistance, including the fall in systolic blood pressure on head-up tilt (ρ=0.46, *P*=.02) and OIR-tilt (ρ=0.63, *P*=.0004) (Figure 6.6) change in systolic blood pressure with isometric exercise (ρ=-0.45, *P*=.03), and PRT after Valsalva manoeuvre (ρ=0.44, *P*=.03), as well as the orthostatic intolerance subscore on the COMPASS-31 (ρ=0.57, *P*=.006), but not with markers of heart rate variability, including ΔHR with isometric exercise, HR_{DB} and Valsalva ratio, DST parameters, and plasma noradrenaline. The total and autonomic p-syn score did not correlate with age, disease duration, intraepidermal, or pilomotor innervation. OIR-tilt correlated with total COMPASS-31 score (ρ=0.32, *P*=.04), suggesting that, as with the AAG patients, the OIR-tilt reflected the severity of autonomic symptoms reported by synucleinopathy patients.

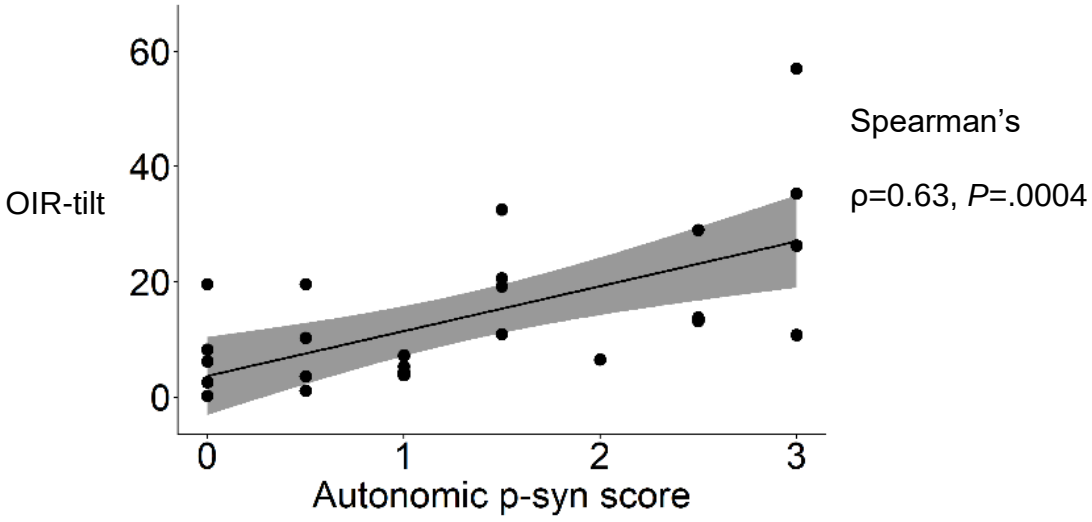


Figure 6.6. Cutaneous mean autonomic p-syn score correlated with orthostatic intolerance ratio on head-up-tilt

6.4 Discussion

This study highlights that cutaneous p-syn has the potential to be a promising diagnostic biomarker in patients presenting with potential pure autonomic failure, particularly in patients with atypical or overlapping clinical features, where both autoimmune and neurodegenerative aetiologies are under consideration. Early pathological confirmation of an underlying synucleinopathy with minimally invasive punch skin biopsy may help avoid unnecessary trials of immune therapy, and its associated costs and complications. P-syn is abundantly present in skin samples from PAF patients, with significantly more p-syn than patients with other synucleinopathies using a semi-quantitative score. Low p-syn scores may be a potential predictor for future clinical phenoconversion to a more widespread alpha-synucleinopathy. A semi-quantitative score for p-syn deposits on autonomic structures in the skin correlated significantly with quantitative cardiovascular biomarkers associated with autonomic control of total peripheral resistance, such as ΔSBP and OIR on tilt, and PRT after Valsalva manoeuvre, as well as orthostatic intolerance symptoms reported by the patients on the COMPASS-31, suggesting that p-syn deposition on peripheral autonomic nerves may lead to dysfunction of autonomic control of total peripheral resistance and symptomatic orthostatic hypotension.

A major clinical challenge is distinguishing between patients with gAChR-negative autoimmune autonomic failure, and neurodegenerative pure autonomic failure at early stages of presentation. As outlined in Chapter 4, patients with PAF tended to be older at presentation, less frequently had urinary retention, and more commonly reported RBD, although this was not always present at initial assessment. Within our cohort of prospective patients, some patients presented at a relatively young age with severe

disabling symptoms with atypical features including patchy anhidrosis, severe gastrointestinal symptoms, and early and severe urogenital failure, leading to trials of immunotherapy with no clinical benefit, and subsequently developed RBD and other features in keeping with an evolving synucleinopathy. In our study, p-syn was present in 100% of patients with PAF and none of the autoimmune and other non-synucleinopathy diseases. In some cases, the biopsies were collected prior to patients developing RBD and other features consistent with an underlying synucleinopathy, suggesting that cutaneous p-syn may be a valuable minimally invasive early diagnostic biomarker, particularly in clinically challenging cases with atypical features.

Patients with PAF had significantly more cutaneous neural p-syn compared to patients with MSA and PD, as measured by a semiquantitative score assessing the presence of p-syn supplying cutaneous autonomic adnexa, subepidermal and dermal nerves and nerve bundles, in keeping with a peripherally predominant α-synucleinopathy. Furthermore, we followed up our patients with PAF for potential phenoconversion, and amongst the patients recruited within the first year of our study, two of the patients presenting with PAF with lower total mean p-syn scores developed signs and symptoms consistent with phenoconversion to a more widespread synucleinopathy within the follow up period of this study, suggesting that a low p-syn score in patients presenting with PAF may be a red flag for future phenoconversion.

We found strong correlations between mean autonomic p-syn score, reflecting deposition of p-syn on nerves supplying cutaneous autonomic adnexa, and quantitative cardiovascular autonomic markers reflecting impaired control of total peripheral resistance, such as the ΔSBP and OIR on tilt, and PRT after Valsalva manoeuvre, as

well as orthostatic intolerance symptoms reported by the patients on the COMPASS-31, but not other cardiovagal, sudomotor, or neurohormonal markers measured in this study. Deposition of abnormally phosphorylated synuclein in the peripheral autonomic nerves may impair their function, leading to a disruption of the autonomic control of peripheral vascular resistance, giving rise to symptomatic orthostatic hypotension.

The patients with α-synucleinopathies demonstrated evidence of impaired postganglionic sudomotor dysfunction and reduced postganglionic autonomic innervation as assessed by mean pilomotor nerve fibre density at the distal leg, with no significant difference between groups. Post-ganglionic sudomotor dysfunction and cutaneous autonomic denervation has previously been demonstrated in MSA,[110] with increasing frequency with more prolonged disease.[102] Our study population was made up of patients referred to a national autonomic centre, including a large proportion with severe and established disease. Given the prolonged disease duration of the patients with MSA at the time of biopsy (median 6 years, IQR 3-8 years), it is unsurprising that we observed loss of the fragile distal unmyelinated cutaneous autonomic nerves, even in patients with presumably more proximal synuclein deposition.

6.4.1 Limitations

One of the main limitations of this study is recruitment was limited to patients referred to a national autonomic unit, meaning the patients studied are likely to have more severe disease, and where there may be diagnostic uncertainty. We had few patients with PD, who are typically managed by local teams and only referred to our centre if they have atypical features or severe autonomic symptoms. We used a simple semi-quantitative method of assessing cutaneous p-syn deposition. The advantage of this simple scoring

method is that it is quick and easy to perform, but we plan to explore further quantitative methods for quantifying p-syn deposition described by other groups.[111]

6.4.2 Summary and future directions

With atypical clinical presentations of autonomic failure, minimally invasive skin biopsies to assess for cutaneous p-syn may help to positively identify a neurodegenerative aetiology and avert trials of unnecessary immune therapy. Patients with PAF had significantly more cutaneous p-syn deposits compared to patients with other α-synucleinopathies, in keeping a peripherally predominant α-synucleinopathy. Autonomic p-syn score correlated significantly with quantitative cardiovascular biomarkers reflecting impaired autonomic regulation of peripheral vascular resistance, suggesting a p-syn deposits may impair peripheral autonomic control of total peripheral resistance and contribute to the pathophysiology of OH. Low levels of p-syn deposits in patients with PAF may be a potential biomarker for phenoconversion, and we plan to continue to follow up our prospective patient cohort to see if this early signal is replicated in larger numbers.

Chapter 7. Cardiovascular autonomic failure correlates with cutaneous autonomic denervation in PD and MSA

7.1 Introduction

Cardiovascular autonomic failure and neurogenic OH are common and disabling features of the neurodegenerative diseases PD and MSA.[112] Severe symptomatic autonomic failure and OH are associated with shortened survival and worse disease progression in both PD and MSA.[113, 114]

Compared to PD, MSA is associated with more rapid disease progression, but overlapping clinical features at presentation may cause initial diagnostic uncertainty. Previous studies have shown biomarkers reflecting postganglionic adrenergic autonomic innervation, like supine plasma noradrenaline levels and cardiac scintigraphy studies, tend to be relatively preserved in MSA compared to PD,[99, 115] suggesting that autonomic failure in MSA occurs due to degeneration and dysfunction of central autonomic networks. However, our group and others have found evidence of postganglionic sudomotor dysfunction and cutaneous somatic and autonomic denervation in MSA, suggesting both central and peripheral degeneration may contribute to the pathophysiology of autonomic failure in MSA.[102-104, 116]

We aimed to evaluate the cardiovascular autonomic profile in patients with MSA and PD, with and without OH, alongside markers of postganglionic denervation, including plasma catecholamines, DST, and cutaneous autonomic and somatic innervation, to characterise the relationship between OH, other quantitative biomarkers of cardiovascular autonomic failure and markers of postganglionic denervation. A secondary aim was to assess

whether these biomarkers could differentiate between MSA and PD, and MSA and PD with autonomic failure.

7.2 Methods

7.2.1 Standard Protocol Approvals, Registrations and Patient Consents

Patients with at least clinically probable PD and MSA according to established consensus criteria were recruited from patients referred to 1) Autonomic Unit, the National Hospital of Neurology and Neurosurgery, London, 2) Neurology Division 'ICS Maugeri' IRCCS of Telese Terme, and 3) Neurology Department, University of Naples Federico II, Naples, between November 2017 and December 2019, as part of a multi-centre natural history and biomarker study in Parkinsonism.[52, 53] The study protocol complied with the Declaration of Helsinki and was approved by the local Institutional Review Boards (Fondazione G. Pascale No. "5/15 Maugeri" and London Bridge Research Ethics Committee, REC reference 16/LO/1656). All subjects gave written informed consent to participate in the study. Study data were collected and managed using REDCap research electronic data capture tools hosted at University College London.[117, 118]

Patients with conditions associated with peripheral neuropathies, including diabetes, HIV, connective tissue disorders and other toxic or metabolic disorders were excluded. Patients with an alternative diagnosis other than PD or MSA after 12-month follow-up were excluded from the final analysis.

7.2.2 Clinical assessment and patient reported outcomes

All patients were assessed by neurologists with movement disorders expertise, with recording of their motor examination and functional impairment using the Hoehn-Yahr scale.[119] Patient reported outcomes were collected the COMPASS-31 and SFN-SIQ.[66, 67] Disease duration was defined as time from onset of motor symptoms to the time of recruitment to the study.

7.2.3 Cardiovascular autonomic testing

7.2.3.1 Core protocol

All patients had a core protocol of cardiovascular autonomic testing performed with recording of blood pressure and heart rate:

1) at rest in the supine position
2) after 1-, 3- and 5-minutes standing
3) before and after isometric exercise (sustained handgrip for 3 minutes at a third of maximum voluntary contraction pressure)
4) with deep breathing, at a rate of 6 breaths/min
5) with the Valsalva manoeuvre (forced expiration at 40mmHg for 10 seconds).

All medications potentially affecting autonomic testing were stopped at least five half-lives prior to testing, and patients were instructed to consume water only for four hours prior to testing. OH was defined as a sustained fall of at least 20mmg systolic or 10mmHg diastolic blood pressure within 3 minutes of standing according to consensus criteria.[14] The Valsalva ratio was calculated by dividing the maximal heart rate developing in response to blood pressure reduction induced by the Valsalva manoeuvre by the minimum heart rate occurring within 30 seconds of the peak heart rate.[56]

7.2.3.2 Comprehensive protocol

In addition, 18 patients studied at the London site also had a 10-minute passive head up tilt to 60°, with recordings of blood pressure and heart rate at 1, 4, 7 and 10 minutes, and blood collected via intravenous catheter in the supine and tilted position for measurement of plasma catecholamines using high performance liquid chromatography.[55] These 18 patients also had beat-to-beat recordings of blood pressure and heart rate with analysis of the blood pressure profile in response to the Valsalva manoeuvre. PRT, a marker of adrenergic control of total peripheral resistance, was defined as the time taken for the systolic blood pressure to recover from phase III of the Valsalva manoeuvre back to the baseline.[56]

In patients who had the comprehensive protocol, we assessed whether OH was neurogenic either by assessment of the blood pressure responses to the Valsalva manoeuvre. In patients assessed with the core protocol, we calculated the change in heart rate over the change in systolic blood pressure at three minutes standing, where $\Delta HR/\Delta SBP < 0.5$ suggests neurogenic OH.[120]

7.2.4 Sudomotor testing

All patients underwent DST at the distal leg bilaterally. DST uses pharmacological stimulation with pilocarpine to directly stimulate the M3 receptors on cutaneous sweat glands, providing an estimation of postganglionic sudomotor function.[63] After iontophoresis with 1% pilocarpine solution, skin was coated with iodine and formation of sweat gland imprints on starch covered tape was recorded. Density of activated sweat glands/cm^3, sweat output/min/cm^3, and average sweat output/gland was calculated.

7.2.5 Morphological analysis

All patients had 3-mm skin biopsies collected from the distal leg bilaterally and processed for indirect immunofluorescence according to standard procedures using a large panel of antibodies, including primary antibodies against collagen type IV (ColIV), protein gene product (PGP) 9.5, a pan-neuronal marker, dopamine-β-hydroxylase (DβH), a marker for noradrenergic fibres, vasoactive intestinal peptide (VIP), a marker for cholinergic fibres, and species-specific secondary antibodies coupled with Cy2 and Cy3 fluorophores.[91] Digital confocal images were acquired using a non-laser confocal system (Apotome2 Zeiss, Jena, Germany, EU).

Intraepidermal nerve fibre (IENF) density was measured according to current guidelines.[69] Quantification of pilomotor and sudomotor nerve fibres using pan-neuronal and selective cholinergic and noradrenergic markers was performed as previously described.[39, 103] For pilomotor nerve fibre quantification, arrector pili muscle segments parallel to the focal plane were acquired using a 20x objective. The single optical section with the most fibres running for at least 100 µm parallel to the major axis of the muscle was selected from the Z-stack. A line was then traced perpendicular to the major axis intercepting the most fibres. Pilomotor nerve fibre density was calculated as the average number of intercepts per muscle width in fibres/mm of all muscles suitable for quantification for each staining for each biopsy. For SNF quantification, Neurolucida 360 software (Microbrightfield Bioscience [MBFB], Williston, VT) was used to identify and trace neural structures in 3D confocal image stacks of sweat glands acquired with a 20x objective. The length of sudomotor nerves measured was divided by the volume of the

imaged gland to give sudomotor nerve fibre density (nm/µm^3). All measurements were performed by the same operator, blinded to subject diagnoses.

For the functional and morphological studies above, the average of both sides was calculated and taken as representative for each patient. Normative data was extracted from a database of 100 healthy volunteers.

7.2.6 Statistical Analysis

Statistical analysis was performed using R, Version 3.6.0. Normality was assessed Shapiro-Wilk tests. Summary data has been presented as median (IQR) as some data was not normally distributed. Patient groups were compared with unpaired two-tailed t-tests/ Mann-Whitney tests, and subgroup comparisons were preformed using ANOVA/Kruskal-Wallis tests with Tukey/Dunn post-hoc tests with Bonferroni corrections for multiple comparisons as appropriate. Chi-squared tests were used to compare categorical data. Correlations were performed using Spearman tests. $P<.05$ was considered significant.

7.3 Results

7.3.1 Demographic and clinical data

The postganglionic sudomotor function, cutaneous autonomic innervation and distribution of neural phosphorylated synuclein of the patients recruited to this multicentre cohort study have been described in previous reports.[48, 104] This study focused on the cardiovascular autonomic testing and the relationship between quantitative autonomic biomarkers and markers of post-ganglionic autonomic denervation autonomic innervation in patients with MSA and PD, with and without OH.

Of 96 patients recruited to the multicentre natural history study, 59 patients had cardiovascular autonomic testing, dynamic sweat testing, and punch skin biopsies performed. Two patients recruited with atypical parkinsonism were subsequently diagnosed with progressive supranuclear palsy at 12-month follow up review and excluded from this study. Fifty-seven patients were included in the final analysis: 37 with PD and 20 with MSA, 16 with parkinsonian subtype and 4 with cerebellar subtype. 32% (18/57) were female and median age was 64 years (IQR 59-70 years) at recruitment. The MSA and PD groups had similar sex distribution, age and disease duration at recruitment, but patients with MSA had greater disease severity as measured by the Hoehn-Yahr scale (4 [3-4] vs 1.5 [1-2], *P*<.001) (Table 7.1). Patients with and without OH were similar in age, but patients with OH had a longer disease duration (33 [20-44] vs 21 [11-23] months, *P*=.005). They reported more severe autonomic symptoms (COMPASS-31 score 33 [25-48] vs 17 [9-30], *P*=.006) and had a higher Hoehn-Yahr grade (3.5 [2.75-4] vs 1 [1-2], *P*=.001) (Table 7.2). Compared to patients without OH, patients with OH had a more prolonged PRT (14.5 [8.9-22.4] seconds vs 2.5 [2.5-2.8] seconds, *P*=.02), attenuated pressor responses to isometric exercise (5 [-2 to 15] mmHg vs 22 [14-26] mmHg, *P*<.001; 4 [1-6] vs 11 [7-14] bpm, *P*=.004), HRDB (7 [4-11] bpm vs 12 [8-18] bpm, *P*=.048) and lower Valsalva ratio (1.10 [1.03-1.18] vs 1.33 [1.21-1.42], *P*=.02), reflecting more widespread sympathetic and parasympathetic autonomic failure. Cardiovascular autonomic biomarkers did not differ significantly between the MSA with OH and PD with OH subgroups (Table 7.3)

7.3.3 Comparison between core and comprehensive cardiovascular autonomic testing

Of the 23 patients with OH on standing, 15 patients had assessment of BP profile with the Valsalva manoeuvre, and all 15/15 (100%) had reduced or absent phase II late blood pressure recovery, phase IV overshoot and prolonged PRT, consistent with impaired adrenergic function and neurogenic OH. In the 8 patients with OH assessed with the core protocol who did not have beat-to-beat monitoring, heart rate rise by 3 minutes stand was minimal, again in keeping with neurogenic OH (median ΔHR 1 [IQR -2 to 4] bpm; median ΔHR/ΔSBP -0.04 (IQR -0.05 to 0.21, ΔHR/ΔSBP <0.5 indicates neurogenic OH).[120]

Of the 20 patients without OH, three patients had assessment of blood pressure profile with the Valsalva manoeuvre, and of these, one patient had an abnormal blood pressure response to the Valsalva manoeuvre, indicating early adrenergic failure (see Case 2 in Box 7.1).

The fall in systolic blood pressure on standing correlated well with the fall in systolic blood pressure with passive head-up tilt, with the best correlation at 5 minutes stand and 4 minutes tilt (ρ=0.91, *P*=4.5 x 10-7). The fall in systolic blood pressure on standing also correlated strongly with PRT after the Valsalva manoeuvre, a marker of adrenergic control of total peripheral resistance (ρ=0.78, *P*=.0003).[57]

Table 7.

7.3.2 Cardiovascular autonomic testing

Supine blood pressure and heart rate were similar amongst the PD and MSA groups (median systolic blood pressure 135 [IQR 124-146] mmHg and median heart rate 65 [IQR 61-71] bpm). Compared to patients with PD, patients with MSA had a significantly greater fall in systolic blood pressure on standing (35 [28-77] vs 10 [1-26] mmHg by 5 minutes, *P*=.004), and reduced blood pressure and heart rate responses to isometric exercise (6 [-1 to 14] vs 25 [20-32] mmHg, *P*<.001; 5 [2-8] vs 9 [4-14] bpm, *P*=.04).

Of the 57 patients studied, 43 patients completed at least 3 minutes of standing challenge, allowing us to assess for sustained OH according to consensus criteria.[14] Of these, 78% (14/18) patients with MSA and 36% (9/25) with PD fulfilled the criteria for OH. The other 14 patients had assessment of blood pressure and heart rate supine and after 1 minute standing only as part of routine clinical testing, and were not included in the analyses comparing patients with and without OH.

Patients with and without OH were similar in age, but patients with OH had a longer disease duration (33 [20-44] vs 21 [11-23] months, *P*=.005). They reported more severe autonomic symptoms (COMPASS-31 score 33 [25-48] vs 17 [9-30], *P*=.006) and had a higher Hoehn-Yahr grade (3.5 [2.75-4] vs 1 [1-2], *P*=.001) (Table 7.2). Compared to patients without OH, patients with OH had a more prolonged PRT (14.5 [8.9-22.4] seconds vs 2.5 [2.5-2.8] seconds, *P*=.02), attenuated pressor responses to isometric exercise (5 [-2 to 15] mmHg vs 22 [14-26] mmHg, *P*<.001; 4 [1-6] vs 11 [7-14] bpm, *P*=.004), HR_{DB} (7 [4-11] bpm vs 12 [8-18] bpm, *P*=.048) and lower Valsalva ratio (1.10 [1.03-1.18] vs 1.33 [1.21-1.42], *P*=.02), reflecting more widespread sympathetic and

parasympathetic autonomic failure. Cardiovascular autonomic biomarkers did not differ significantly between the MSA with OH and PD with OH subgroups (Table 7.3)

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Of the 20 patients without OH, three patients had assessment of blood pressure profile with the Valsalva manoeuvre, and of these, one patient had an abnormal blood pressure response to the Valsalva manoeuvre, indicating early adrenergic failure (see Case 2 in Box 7.1).

The fall in systolic blood pressure on standing correlated well with the fall in systolic blood pressure with passive head-up tilt, with the best correlation at 5 minutes stand and 4 minutes tilt (ρ=0.91, P=4.5 x 10^{-7}). The fall in systolic blood pressure on standing also correlated strongly with PRT after the Valsalva manoeuvre, a marker of adrenergic control of total peripheral resistance (ρ=0.78, P=.0003).[57]

Table 7.1. Clinical details and cardiovascular testing in MSA and PD

	Median (IQR)		
	MSA (n=20)	PD (n=37)	*P*-value
Clinical details			
Age, y	63 (55-68)	65 (58-70)	.48
Sex (F/M)	6/14	12/25	>.99
Disease duration, mo	30 (17-42)	21 (12-23)	.10
Hoehn-Yahr	4 (3-4)	1.5 (1-2)	<.001
COMPASS-31	32 (16-45)	18 (11-29)	.12
SFN-SIQ	9 (8-10)	6 (4-10)	.26
Cardiovascular testing			
Δ SBP on standing			
1 min, mmHg	32 (14-41)	7 (1-20)	.03
3 min, mmHg	32 (19-65)	4 (-2 to 19)	.004
5 min, mmHg	35 (28-77)	10 (1-26)	.004
Isometric exercise			
Δ SBP, mmHg	6 (-1 to 14)	25 (20-32)	<.001
Δ HR, bpm	5 (2-8)	9 (5-14)	.04
HR_{DB}, bpm	8 (6-11)	12 (8-18)	.06
Valsalva ratio	1.20 (1.12-1.3)	1.19 (1.08-1.39)	.89
Pressure recovery time, s	14.5 (7.4-23.6)	7.6 (2.8-14.6)	.30
Supine noradrenaline, pg/ml	256 (240-303)	216 (158-223)	.02
Δ noradrenaline on tilt, pg/ml	11 (5-12)	67 (38-71)	.05

SBP, systolic blood pressure; HR, heart rate

Table 7.2. Clinical and cardiovascular testing in patients with and without OH

	Median (IQR)		*P*-Value
	OH (n=23)	No OH (n=20)	
Clinical details			
Age, y	64 (56-71)	63 (60-69)	.90
Sex (F/M)	5/18	9/11	.20
Disease duration, months	33 (20-44)	21 (11-23)	.002
Hoehn-Yahr	3.5 (2.75-4)	1 (1-2)	<.001
COMPASS-31	33 (25-48)	17 (9-30)	.006
SFN-SIQ	10 (7-11)	7 (5-10)	.16
Cardiovascular testing			
Δ SBP on standing			
1 min, mmHg	37 (19-56)	1 (-4-16)	<.001
3 min, mmHg	37 (21-70)	4 (-2 to 9)	<.001
5 min, mmHg	35 (29-74)	4 (-1 to 11)	<.001
Isometric exercise			
Δ SBP, mmHg	5 (-2[a] to 15)	22 (14-26)	<.001
Δ HR, bpm	4 (1-6)	11 (7-14)	.004
HR_{DB}, bpm	7 (4-11)	12 (8-18)	.048
Valsalva ratio	1.10 (1.03-1.18)	1.33 (1.21-1.42)	.02
Pressure recovery time, s	14.5 (8.9-22.4)	2.7 (2.5-2.8)	.02
Supine noradrenaline, pg/ml	242 (228-293)	232 (224-239)	.72
Δ noradrenaline on tilt, pg/ml	11 (6-18)	93 (80-106)	.06

SBP, systolic blood pressure; HR, heart rate

Table 7.3. Subgroup comparisons

	Median (IQR)				*P*-Value	
Variable	MSA+OH (n=16)	MSA, no OH (n=4)	PD+OH (n=9)	PD, no OH (n=16)	ANOVA	MSA+OH v PD+OH
Clinical details						
Age, y	64 (55-68)	62 (59-66)	65 (58-74)	64 (60-69)	.91	.81
Sex (F/M)	3/13	1/3	2/7	8/8	.35	
Disease dur., mo	39 (29-59)	16 (10-20)	23 (19-35)	21 (11-23)	.009	>.99
Hoehn-Yahr	4 (3.5-4)	3 (2-3)	3 (2-3)	1 (1-2)	<.001	.45
SFN-SIQ	10 (8-10)	9 (9-9)	13 (6-21)	7 (5-11)	.54	>.99
COMPASS-31	33 (22-48)	22 (17-26)	36 (28-48)	18 (9-31)	.07	>.99
Cardiovascular testing						
Δ SBP-on standing						
1 min, mmHg	38 (28-51)	-4[a] (-9 to 8)	21 (6-59)	3 (-3[a] to 16)	.001	.87
3 min, mmHg	37 (30-75)	5 (-2 to 10)	21 (19-58)	4 (-2[a] to 7)	<.001	.95
5 min, mmHg	40 (30-84)	-2[a] (-4 to 8)	29 (23-44)	5 (1-11)	<.001	.59
Isometric exercise						
Δ SBP, mmHg	5 (-2.5[a] to 10.2)	11 (5-16)	10 (3-21)	25 (22-27)	<.001	.56
Δ HR, bpm	7 (3-6)	7 (3-12)	1 (0-4)	12 (8-14)	.001	>.99
HR_{DB}, bpm	7 (4-9)	13 (11-16)	12 (4-17)	11 (8-18)	.09	.75
Valsalva ratio	1.20 (1.13-1.28)	1.33 (1.29-1.34)	1.14 (1.07-1.19)	1.37 (1.21-1.50)	.09	>.99
Pressure recovery time,[b] s	14.5 (7.4-23.6)	NA	17.2 (14.6-19.7)	2.7 (3.5-2.8)	.08	>.99
Supine NA,[c] pg/ml	256 (240-303)	NA	158 (154-190)	232 (224-239)	.04	.04
Δ NA on tilt, pg/ml	11 (5-12)	NA	38 (22-55)	93 (80-106)	.08	.81
DST						
Glands/cm^3	24 (19-40)	39 (25-46)	53 (41-55)	46 (34-57)	.04	.05
Output/gland, nL	3.1 (1.5-4.3)	4.6 (3.2-5.1)	3.0 (2.5-4.1)	2.7 (2.2-4.7)	.85	.95
Sweat output, nL/cm^3	73 (26-145)	79 (22-166)	149 (119-194)	119 (38-190)	.25	.31
Skin biopsies						
IENFD, f/mm	1.8 (0.9-4.5)	5.0 (4.9-10.3)	5.9 (2.6-9.1)	7.8 (6.1-9.4)	.005	.23
Pilomotor nerve fibre density, f/mm						
PGP	28.8 (20.0-46.7)	52.6 (50.2-59.0)	45.6 (38.2-50.7)	53.7 (39.5-59.0)	.02	.77
VIP	0.0 (0.0-5.2)	25.2 (21.6-25.8)	6.6 (4.3-13.0)	9.8 (5.7-15.2)	.004	.30
DβH	4.5 (0-13.6)	42.0 (38.3-43.9)	21.6 (11.9-40.2)	33.6 (20.4-45.2)	.01	.35
Sudomotor nerve fibre density, nm/μm^3						
PGP	0.7 (0.5-2.0)	1.9 (1.6-2.7)	3.1 (2.1-3.7)	2.8 (2.5-3.9)	.02	.13
VIP	0.6 (0.0-0.9)	1.3 (1.2-1.4)	1.3 (0.7-1.6)	1.6 (1.4-1.8)	.006	.54

SBP, systolic blood pressure; HR, heart rate; NA, noradrenaline

7.3.4 Plasma noradrenaline

Seventeen patients had plasma noradrenaline levels measured: 12 with MSA, all of whom had OH, and five patients with PD, three with OH and two without OH. Patients with MSA had normal supine noradrenaline (256 [240-303] pg/ml), with minimal rise on tilt (11 [5-12] pg/ml). In comparison, patients with PD with OH had significantly lower supine noradrenaline (158 [154-190] pg/ml, *P*=.04), with modest rise on tilt (38 [22-55] pg/ml). Patients with PD without OH had normal supine noradrenaline (232 [224-239] pg/ml) and preserved rise on tilt (93 [80-106] pg/ml) (Figure 7.1).

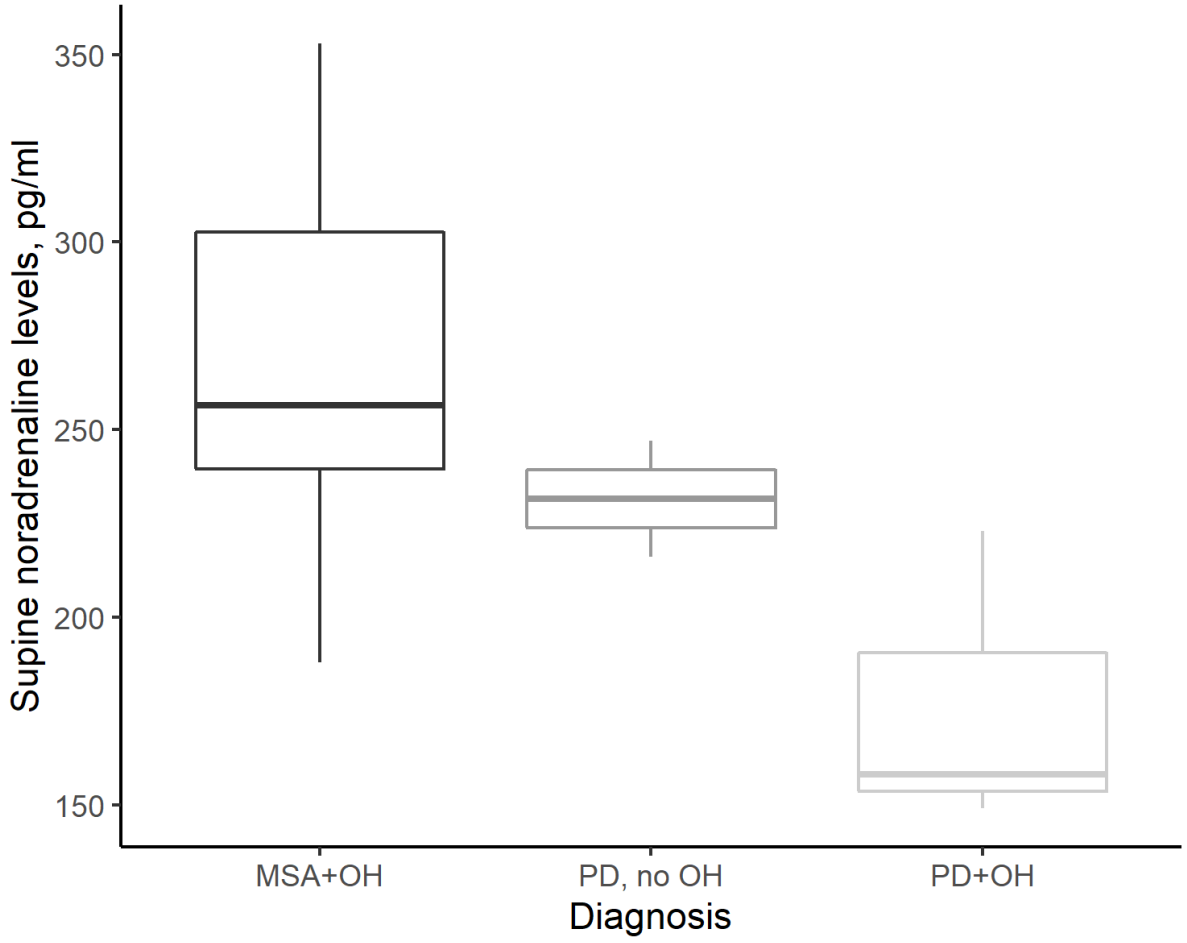

Figure 7.1. Supine noradrenaline levels in MSA and PD, with and without OH. Supine noradrenaline levels were significantly lower in the PD+OH group compared to the MSA group (P=.04), with intermediate results in the PD without OH group.

7.3.5 Postganglionic sudomotor function and cutaneous somatic and autonomic innervation

Our group recently reported that postganglionic sudomotor function and cutaneous sudomotor innervation was reduced in patients with MSA and PD compared to healthy controls, with greater impairments in patients with MSA.[104] In this subset of patients who had cardiovascular autonomic testing, we had similar results, with more impaired sweat production/cm^3, sweat output/gland, and sweat gland density, and cholinergic sudomotor innervation in patients with MSA and PD compared to controls, with the MSA patients showing reduced sweat gland density and cholinergic sudomotor innervation compared to patients with PD (Table 7.4). There was no difference in postganglionic sudomotor function between patients with and without OH (Table 7.5).

Overall, somatic and autonomic innervation at the distal leg was significantly reduced in patients with OH compared to controls ($P \leq .001$), with intermediate levels seen in the no OH group. Intraepidermal nerve fibre density was reduced in OH (2.7 [1.3-5.4] fibres/mm, and non-OH groups (7.4 [5.1-10.0] fibres/mm) compared to controls (12.1 (10.5-14.0 fibres/mm, $P < .001$), without significant difference between the OH and no OH groups ($P = .25$). Pilomotor adrenergic innervation was lowest in the OH group (10.6 [0.4-21.4] fibres/mm), followed by the non-OH group (36.1 [28.4-45.7] fibres/mm) then the control group (52 [49.5-55] fibres/mm), with a significant difference between the OH and control group ($P \leq .001$) (Figure 7.2). Sudomotor cholinergic innervation was lowest in the OH group (0.9 [0.1-1.3] fibres/mm), followed by the non-OH group (1.6 [1.4-1.8] fibres/mm) then the control group (2.1 [1.9-2.7] fibres/mm), with significant differences between all groups ($P \leq .01$).

Table 7.4 Postganglionic sudomotor function and cutaneous innervation in MSA and PD

	Median (IQR)			*P*-value			
	MSA (n=20)	PD (n=37)	CTRL (n=100) †	ANOVA	CTRL vs MSA	CTRL vs PD	MSA vs PD
Clinical details							
Age, y	63 (55-68)	65 (58-70)	61 (54-67)	.22			
Sex (F/M)	6/14	12/25	49/51	.10			
DST							
Glands/cm^3	28 (18-45)	46 (37-55)	66 (62-85)	<.001	<.001	<.001	.007
Output/gland, nL	3.1 (1.4-4.3)	3.5 (2.5-4.7)	11.4 (7.7-13.2)	<.001	<.001	<.001	.09
Sweat output, nL/cm^3	73 (22-139)	153 (102-261)	591 (363-871)	<.001	<.001	<.001	.06
Skin biopsies							
IENFD, f/mm	3.3 (1.4-5.0)	7.8 (5.3-8.7)	12.1 (10.5-14.0)	<.001	<.001	<.001	.22
PNFD, f/mm							
PGP	37.8 (22.0-50.6)	50.7 (38.2-65.4)	70 (66-90)	<.001	<.001	<.001	.21
VIP	0.8 (0.0-17.9)	8.1 (2.6-21.5)	60.3 (52.4-69.4)	<.001	<.001	<.001	.59
DβH	10.6 (0.4-30.3)	33.6 (20.4-44.1)	52 (49.5- 55)	<.001	<.001	<.001	.18
SNFD, nm/µm^3							
PGP	1.2 (0.6-3.2)	2.9 (2.3-3.7)	3.6 (3.1-3.9)	<.001	<.001	.13	.03
VIP	0.8 (0.3-1.2)	1.6 (1.1-1.9)	2.1 (1.9-2.7)	<.001	<.001	<.001	.02

PNFD, pilomotor nerve fibre density; SNFD, sudomotor nerve fibre density.

Table 7.516 Postganglionic sudomotor function and cutaneous innervation in patients with and without OH

Variable	Median (IQR)			*P*-value			
	OH (n=23)	No OH (n=20)	CTRL (n=100)	ANOVA	CTRL vs OH	CTRL vs No OH	No OH vs OH
DST							
Glands/cm^3	37 (21-50)	45 (30-52)	66 (62-85)	<.001	<.001	<.001	.59
Output/gland, nL	3.0 (2.3-4.3)	3.0 (2.2-4.9)	11.4 (7.7-13.2)	<.001	<.001	<.001	.93
Sweat output, nL/cm^3	113 (62-166)	119 (28-190)	591 (363-871)	<.001	<.001	<.001	1
Skin biopsies							
IENFD, f/mm	2.7 (1.3-5.4)	7.4 (5.1-10.0)	12.1 (10.5-14.0)	<.001	<.001	<.001	.25
PNF, f/mm							
PGP	36.6 (23.6-48.4)	53.1 (44.2-61.1)	70 (66-90)	<.001	<.001	.01	.09
VIP	2.8 (0-7.5)	11.8 (6.5-23.4)	60.3 (52.4-69.4)	<.001	<.001	.002	.13
DβH	10.6 (0.4-21.4)	36.1 (28.4-45.7)	52 (49.5- 55)	<.001	<.001	.06	.08
SNF, nm/µm^3							
PGP	1.9 (0.7-3.6)	2.8 (2.2-3.7)	3.6 (3.1-3.9)	.001	.001	.20	.46
VIP	0.9 (0.1-1.3)	1.6 (1.4-1.8)	2.1 (1.9-2.7)	<.001	<.001	.01	.002

PNFD, pilomotor nerve fibre density; SNFD, sudomotor nerve fibre density.

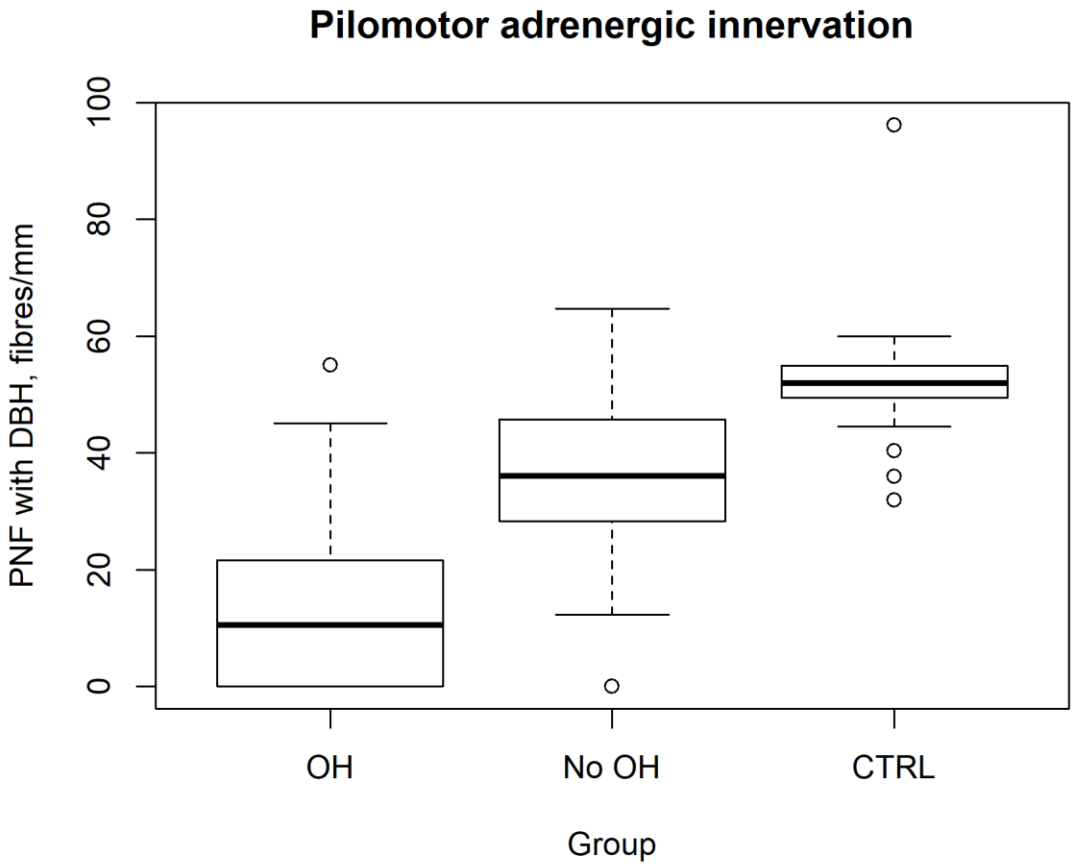

Figure 7.2. Comparison of pilomotor adrenergic innervation between patients with and without orthostatic hypotension and controls. Pilomotor adrenergic innervation was lowest in the OH group (10.6 [0.4-21.4] fibres/mm), followed by the non-OH group (36.1 [28.4-45.7] fibres/mm) then the control group (52 [49.5-55] fibres/mm), with a significant difference between the OH and control group (P≤.001)

7.3.6 Correlations between OH, cutaneous autonomic denervation, patient reported outcomes, disease severity, and disease duration

There was a strong correlation between the severity of OH as measured by the fall in systolic blood pressure at 5 minutes standing and pilomotor adrenergic innervation (ρ=-0.54, *P*=.003). The fall in systolic blood pressure on standing also correlated significantly with patient reported autonomic symptoms on the COMPASS-31 (ρ=0.55, *P*=.002), and disease severity as measured by the Hoehn-Yahr scale (ρ=0.50, *P*=.004) (Figure 7.3).

Disease duration correlated with the fall in systolic blood pressure at on standing (ρ=0.59, *P*<.001), intraepidermal innervation (ρ=-0.41, *P*=.002), pilomotor adrenergic innervation, ρ=-0.41, *P*=.006), sudomotor cholinergic innervation, ρ=-0.51, *P*<.001), and Hoehn-Yahr grade (ρ=-0.46, *P*=.001).

Supine noradrenaline did not correlate with any quantitative cardiovascular or sudomotor testing parameters or cutaneous innervation.

Box 7.1. Illustrative clinical cases

1. A 66-year-old man with MSA, neurogenic OH and cardiovascular autonomic failure had a normal supine plasma noradrenaline level (224pg/ml) and cardiac MIBG suggesting preserved postganglionic innervation. However, we found severe postganglionic sudomotor dysfunction and cutaneous somatic and autonomic denervation (Figure 7.4). Our study findings and this case highlight how postganglionic autonomic denervation may not occur in a uniform fashion within the disease cohort and individual patients.
2. A 55-year-old man with a 2-year history of PD, longstanding constipation, and occasional episodes of orthostatic intolerance increasing over the previous year had a minimal fall in blood pressure on standing with good compensatory heart rate rise (9/7mmHg, 25bpm), preserved pressor responses with isometric exercise (22mmHg; 16bpm), HR_{DB} (12bpm) and Valsalva ratio (1.92). However, beat-to-beat blood pressure recording during the Valsalva manoeuvre showed reduced phase II late recovery and phase IV overshoot indicating early adrenergic failure. ~~We suggest that the core protocol may be useful to screen for cardiovascular autonomic failure in patients with α-synucleinopathies, but if results are equivocal, patients with evolving symptoms should undergo comprehensive testing at a specialist autonomic laboratory.~~

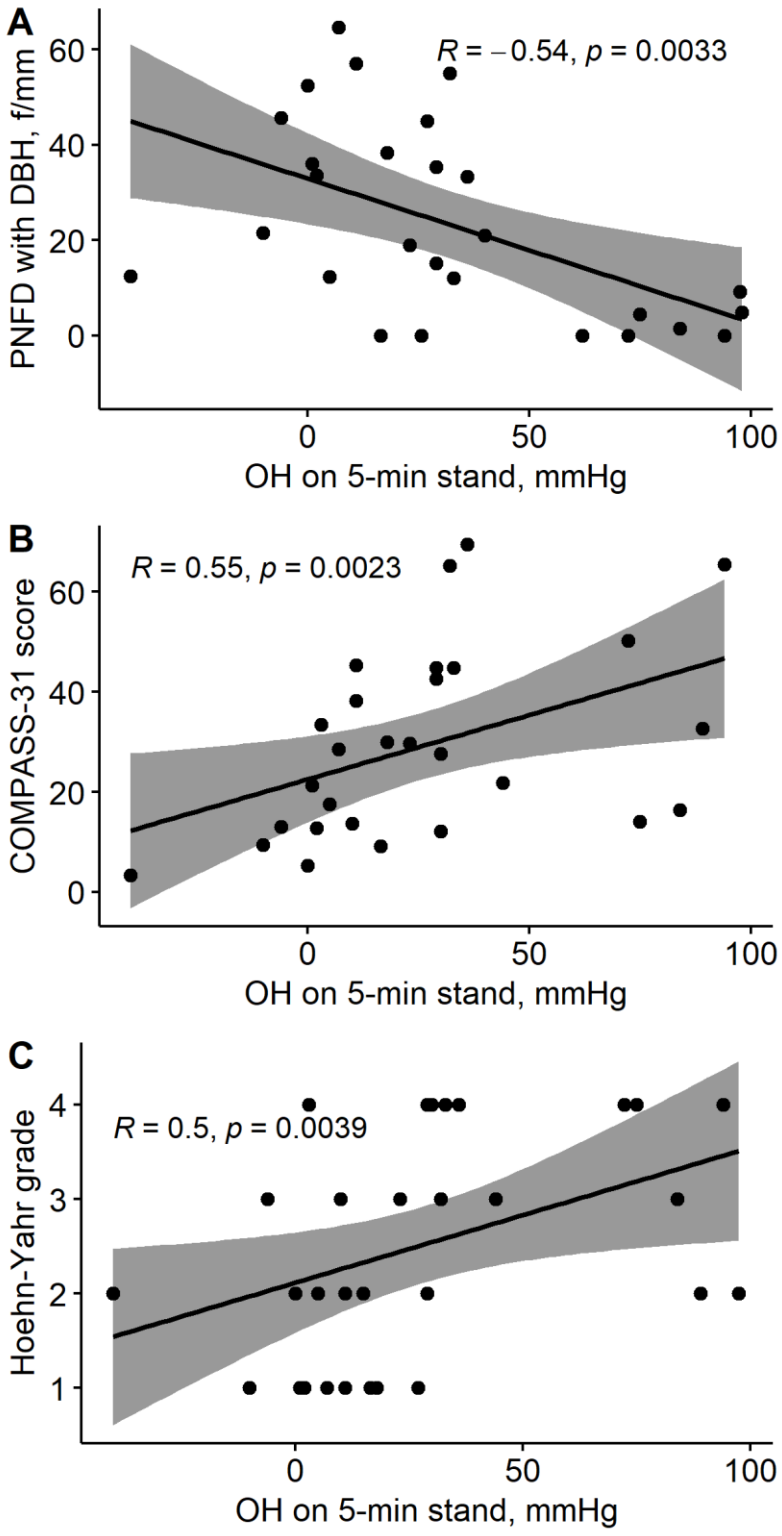

Figure 7.3. Severity of OH correlated with cutaneous adrenergic denervation, patient reported symptoms, and disease severity. Severity of OH as measured by the fall in systolic blood pressure on standing correlated with pilomotor nerve fibre density with adrenergic marker DβH (A). OH severity also correlated with patient reported autonomic symptoms on the COMPASS-31 questionnaire (B) and disease status as measured by the Hoehn-Yahr scale (C).

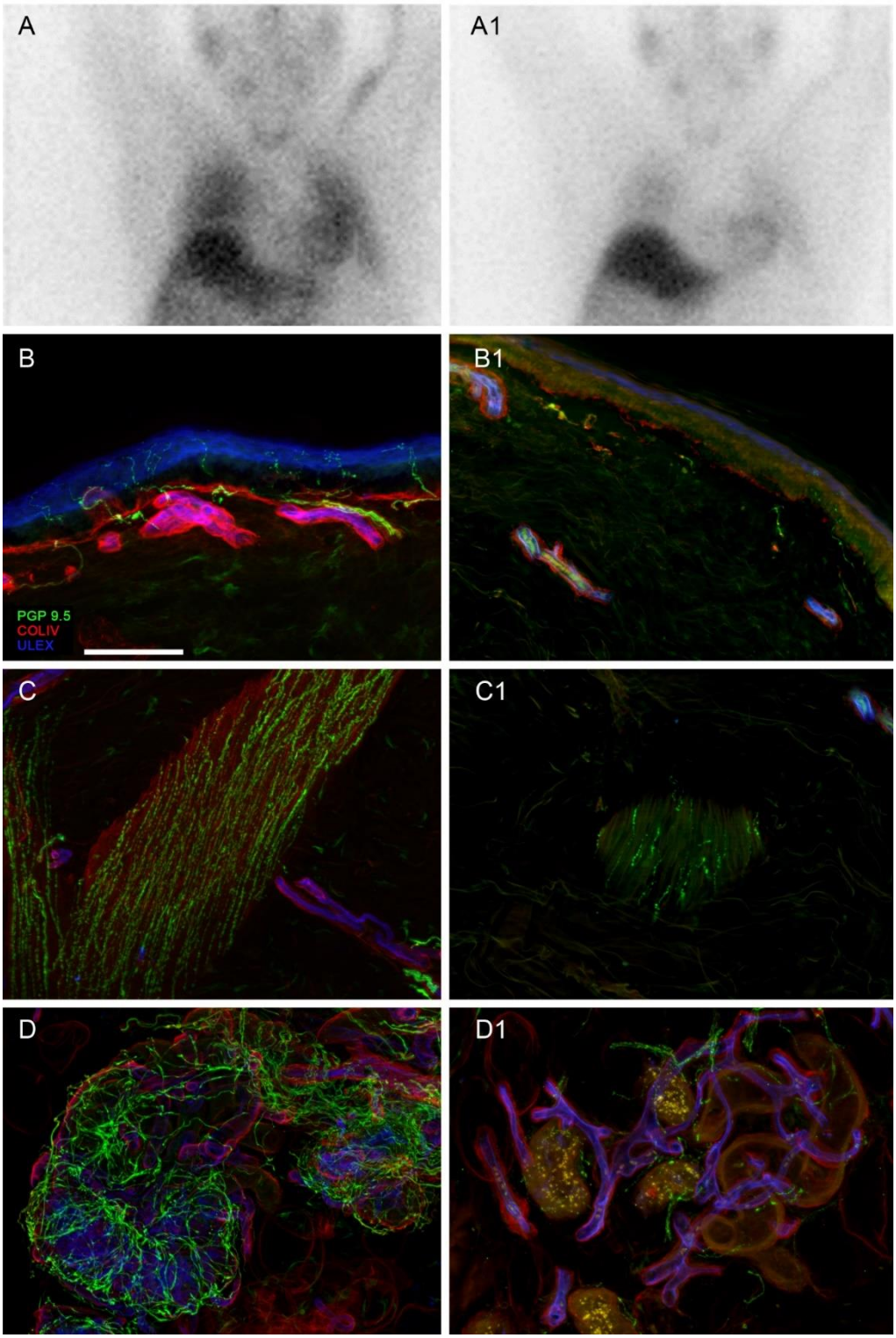

Figure 7.4. Cardiac MIBG and cutaneous innervation in a patient with MSA (Case 1). MIBG study performed as part of clinical work up. Images acquired 10 minutes (A) and 4 hours (A1) post injection showed good tracer uptake in the myocardium with a heart to mediastinum ratio of 1.73, indicating preserved postganglionic innervation. However, punch skin biopsies from the distal legs bilaterally revealed severe cutaneous denervation with marked loss of intraepidermal (0.1-1.1 fibres/mm), pilomotor (15.2-32.0 fibres/mm) and sudomotor fibres (0.4 nm/µm3) with pan-neuronal marker PGP (B-D, control; B1-D1, patient), and a complete loss of pilomotor and sudomotor fibres with cholinergic marker VIP (not shown here). Scale bar: 100µm.

7.4 Discussion

Our study has revealed insights into OH and cardiovascular autonomic failure in MSA and PD with clinically relevant implications. In our cohort of patients with MSA and PD, OH does not occur in isolation, but in the context of more generalised impairment of other autonomic reflexes, in keeping with more widespread cardiovascular autonomic failure. OH was associated with cutaneous adrenergic denervation, with significant correlations between the severity of OH, cutaneous adrenergic denervation, patient reported symptoms, and Hoehn-Yahr grade, and no differences between subgroups of MSA and PD with OH. To our knowledge, this is the first study demonstrating an association between cutaneous adrenergic denervation and orthostatic hypotension in PD and MSA. While cutaneous adrenergic denervation is unlikely to contribute significantly to impairment of systemic blood pressure regulation, we hypothesize that it may reflect more widespread peripheral adrenergic denervation. In healthy individuals, a series of central and peripheral autonomic networks are activated to maintain blood pressure and organ perfusion on orthostasis. In MSA, the underlying pathophysiology of neurogenic orthostatic hypotension has previously been thought to be predominantly through dysfunction of central autonomic networks, in contrast to dysfunction of peripheral postganglionic autonomic nerves in PD. Our study suggests postganglionic adrenergic denervation may contribute to the pathophysiology of OH in patients with MSA as well as patients with PD, with potential impact on responses to therapeutic agents. Furthermore, our results confirm previous studies showing OH contributes significantly to functional disability in PD and MSA.[99]

We found a reduction in cutaneous adrenergic pilomotor innervation in patients with OH compared to healthy controls, with no significant differences between the MSA with OH and PD with OH subgroups on post-hoc analysis. Our results are in keeping with a previous study by Gibbons et al, who compared the cutaneous innervation of 28 patients with PD with and without autonomic failure to 23 healthy controls and found patients with autonomic failure had reduced pilomotor and sudomotor innervation compared to controls. Our results may appear to contradict the study from Donadio et al who reported that cutaneous synuclein deposits are mainly found in somatic fibres in MSA-P and within autonomic fibres in PD with OH, with skin innervation mirroring p-syn deposition.[46] However, closer analysis of the supplementary data from their study shows intraepidermal and sudomotor innervation at the distal leg was reduced both MSA-P and PD+OH patients compared to controls, with pilomotor innervation reduced in PD+OH compared to both MSA-P and controls. Our cohort of patients with PD was significantly younger than theirs (median 65 [IQR 58-74] years compared to mean 74 ± SD 6.7 years) and we studied autonomic innervation using pan-neuronal, cholinergic, and adrenergic specific markers, so the studies are not directly comparable.

Post mortem studies have shown a loss of sympathetic preganglionic cells in the intermediolateral cell column of the thoracolumbar spinal cord in PD and MSA patients with autonomic failure compared to patients without autonomic failure and normal controls, [13] but a loss of postganglionic cardiac adrenergic fibres in PD patients only.[106] In vivo cardiac scintigraphy studies have also demonstrated cardiac uptake of noradrenaline analogues tends to be spared in MSA compared to PD,[100, 115, 121] although these studies do not always reliably distinguish between individuals with MSA and PD.[99,

[101] The differences in noradrenergic innervation at other bodily sites as assessed by ^{18}F-dopamine positron emission topographic scans and postmortem neurochemical data suggests that the difference between patients with Lewy body synucleinopathies, including patients with PD, and MSA is cardioselective; and there were no differences between norepinephrine levels in the sympathetic ganglia and a number of other organs studied.[122] In our study, cardiovascular autonomic testing did not differ significantly between MSA with OH and PD with OH subgroups, like previous reports,[99, 123] suggesting that cardiovascular autonomic testing on its own cannot reliably distinguish between patients with MSA with OH and PD with OH. Interestingly, while there were no significant subgroup differences, the MSA with OH patients had the most severe OH with standing and diminished blood pressure response to isometric exercise, whereas the PD with OH patients had the most diminished heart rate response to isometric exercise and lowest Valsalva ratio, perhaps reflecting greater cardiac adrenergic denervation.

In our study, supine noradrenaline levels were significantly higher in MSA vs PD with OH subgroups, in keeping with previous studies,[59, 124] and in fact, supine noradrenaline was the only biomarker to differ significantly amongst the MSA and PD with OH subgroups. The relationship between plasma noradrenaline levels and sympathetic innervation and activity is complex, and resting plasma supine noradrenaline levels are likely to reflect both production from postganglionic pre-synaptic terminals, cellular reuptake, metabolism and storage. Peripheral adrenergic denervation is likely to contribute to abnormal responses to physiological and pharmacological stimuli, including novel therapeutic agents for OH that aim to enhance availability of noradrenaline.[125-129] Previous studies using surrogate markers of postganglionic autonomic denervation to try predict

responses to novel agents for OH have generated conflicting results. Palma et al's study of 20 patients with OH found supine noradrenaline levels predicted pressor response to droxidopa, a pro-drug of noradrenaline, whereas Shibao at al's larger study of 99 patients with OH found neither plasma noradrenaline nor cardiac innervation on MIBG were good predictors for response to noradrenaline reuptake inhibitor atomoxetine.[126, 128] In our study, plasma noradrenaline did not correlate with cutaneous adrenergic innervation, suggesting it may be oversimplistic to use plasma noradrenaline as a single biomarker to estimate global postganglionic adrenergic denervation.

The core cardiovascular autonomic testing protocol used in this multicentre study is of interest as it can be performed without specialist equipment for beat-to-beat blood pressure recording, potentially enabling more patients to access objective assessments of autonomic function. Quantitative markers of cardiovascular autonomic failure on the core protocol correlated well with comprehensive testing performed in a subset of patients, as well as disease severity and patient reported symptoms. However, one of three patients we studied without OH on stand (Case 2) who had preserved responses to the rest of the core protocol had abnormal blood pressure response to the Valsalva manoeuvre suggesting early adrenergic failure. The core protocol may be useful to screen for cardiovascular autonomic failure in patients with α-synucleinopathies, but if results are equivocal, patients with evolving symptoms should undergo comprehensive testing at a specialist autonomic laboratory.

In this exploratory multicentre study, we chose to study 3-mm skin distal leg biopsies, for which there is excellent safety data previously reported, with no serious side effects reported in over 35000 biopsies performed over 15 years across 10 laboratories.[69] We

chose to quantify cutaneous sudomotor and pilomotor autonomic innervation as our group and others have previously established and validated methods to quantify innervation to these autonomic adnexa.[39, 40, 69] The variability and complexity of cutaneous vasculature and its innervation has limited previous attempts to quantify vascular innervation. Sohn et al recently outlined a method of quantifying cutaneous vessel innervation in 20 diabetic patients and 19 controls, and had similar findings to our study, showing individuals with OH had lower vascular innervation than those without OH, with significant correlations between neurovascular density and cardiovascular autonomic biomarkers, including blood pressure drop during head-up tilt and blood pressure overshoot on phase VI of the Valsalva manoeuvre, and autonomic symptom scores.[130]

One of the strengths of our study is that patients were systematically assessed with combination of cardiovascular and sudomotor testing; with good correlations seen between the findings on the core testing protocol and the comprehensive protocol performed on a subset of patients seen at the London site. As the London site, was a national autonomic referral centre, most patients had OH and cardiovascular autonomic failure. Relatively few patients without OH were studied with the comprehensive protocol. We plan to extend the current study by recruiting further patients with and without OH and longitudinally evaluating the progression of the biomarkers we have studied. One of the limitations of our study is that none of the patients underwent post-mortem to confirm the clinical diagnosis, and there is overlap between the clinical criteria of MSA and PD with OH. Nevertheless, all patients were assessed by neurologists with movement

disorders and autonomic expertise and followed up for two years from initial recruitment, with exclusion of subjects who had an alternative clinical diagnosis on follow up.

In summary, in our cohort of patients with PD and MSA, OH was associated with cutaneous adrenergic denervation, with significant corelations between severity of OH, cutaneous adrenergic denervation, patient reported symptoms and Hoehn-Yahr grade. Postganglionic autonomic denervation may contribute to the pathophysiology of autonomic failure in both PD and MSA and influence response to therapeutic agents for OH.

Chapter 8. Conclusion

8.1 Thesis synopsis

The overarching aim of this PhD was to develop multimodal objective quantitative biomarkers to improve the diagnosis and treatment of patients with primary autonomic failure due to autoimmune and neurodegenerative pathology. This final chapter summarises the outcomes of my research to date, identifies limitations of the current work and outlines future questions that should be explored to further advance the field.

8.1.1 Multimodal biomarkers in AAG (Chapter 3)

Firstly, we aimed to use panel of multiple autonomic biomarkers to capture the full phenotype of patients with gAChR-positive AAG and define biomarkers to quantify response to immune therapy. We demonstrated these patients had a characteristic phenotype of widespread parasympathetic and sympathetic autonomic failure, with prominent cholinergic deficits. We demonstrated significant improvements with immune therapy with quantitative biomarkers reflecting sympathetic and parasympathetic autonomic function, patient reported outcomes, and cutaneous innervation, demonstrating that clinically meaningful recovery was possible even in longstanding disease with evidence of postganglionic denervation. We defined a newcomposite marker of orthostatic intolerance, the OIR-tilt, which correlated with patient reported autonomic symptoms and physical disability at baseline. Improvements in OIR-tilt correlated with improvements in patient reported outcomes, suggesting it was a sensitive biomarker that reflected both the severity of patient symptoms at baseline and improvements following immunotherapy.

Following the presentation of our findings at national and international meetings and publication in the Annals of Neurology (Appendix 1), the NHS England Immunoglobulin Expert Working Group have included autoimmune autonomic ganglionopathy as an indication for routine commissioning for therapeutic immunoglobulin, outlining supportive clinical features, treatment strategies and outcome measures to monitor effects of treatment, incorporating results from our work (Appendix 2). Our approach of using multi-modal quantitative autonomic biomarkers with patient reported outcomes has been discussed in subsequent commentaries and reviews,[2, 3] and adopted in subsequent research studies from our centre and others worldwide. [4, 5, 131]

8.1.2 Multimodal biomarkers differentiate gAChR-positive and gAChR-negative autoimmune autonomic failure and PAF (Chapter 4)

We then asked whether deep phenotyping with multimodal biomarkers could differentiate between gAChR-positive and gAChR-negative autoimmune autonomic failure and PAF. Whilst gAChR-positive patients had a distinct consistent phenotype, we found gAChR-negative patients had a heterogenous phenotype, including patients with widespread autonomic failure similar to the gAChR-positive group, as well as patients with parasympathetic, cholinergic predominant autonomic failure, and sympathetic adrenergic predominant autonomic failure, at times associated with a peripheral sensorimotor neuropathy or sensory neuronopathy.

We also asked which features could help to distinguish between gAChR-negative autoimmune autonomic failure and PAF. A younger patient with a history of antecedent events prior to developing autonomic failure, other autoimmune diseases, urinary retention requiring catheterisation, combined parasympathetic and sympathetic pupillary

deficits, and a large fibre neuropathy or neuronopathy were indicators of possible gAChR-negative autoimmune autonomic failure, whereas an older patient presenting with RBD, supine hypertension, more severe OH, low HR_{DB} and supine noradrenaline levels was more likely to have PAF.

Given the differences in clinical phenotype in gAChR-positive and gAChR-negative autoimmune autonomic failure patients, we hypothesised we would also find differences in their cutaneous somatic and autonomic innervation. We commonly observed highly fragmented and beaded cutaneous nerves in gAChR-positive patients, possibly reflecting downstream impaired axoplasmic transport distal to a ganglionic pathology. Compared to gAChR-positive patients, gAChR-negative patients demonstrated significantly greater adrenergic pilomotor denervation, in keeping with the sympathetic predominant autonomic failure seen in most of the cohort, suggesting a different pathophysiological process with preferential loss of postganglionic adrenergic fibres. Follow up biopsies showed striking improvements mirroring the clinical recovery after immune therapy.

8.1.3 Multimodal autonomic biomarkers differentiate PAF, MSA and LBD and predict phenoconversion in PAF (Chapter 5)

We then explored whether multimodal autonomic biomarkers could differentiate between chronic progressive autonomic failure syndromes due to deposition of α-synuclein with a large retrospective study of patients seen over the last twenty years in a single autonomic referral centre. At first assessment, patients with PAF typically had severe OH, low Valsalva ratio and supine noradrenaline levels, and sympathetic pupillary deficits, suggesting greater postganglionic adrenergic dysfunction. Patients with MSA were younger at first presentation, frequently had urinary retention requiring

catheterisation, had higher supine heart rates, supine plasma noradrenaline and normal pupillary function, suggesting relatively preserved postganglionic function. In keeping with the objective autonomic testing, patients with PAF reported significantly greater orthostatic intolerance on standardised questionnaires, but similarly severe levels of physical disability compared to patients with MSA, suggesting severe symptomatic OH has a significant impact on quality of life, even without any motor symptoms.

Amongst patients diagnosed with PAF at initial assessment, normal pupils, supine noradrenaline ≥ 200pg/ml, preserved HR_{DB} ≥ 10bpm, and OIR-tilt < 6 at first assessment were all significantly predictors of phenoconversion to MSA or LBD, with younger age at presentation and higher supine noradrenaline associated with conversion to MSA rather than LBD.

8.1.4 Cutaneous p-syn differentiates PAF from non-synucleinopathy related diseases and other α-synucleinopathies (Chapter 6)

In this study, we first explored whether the presence of cutaneous p-syn deposits could differentiate between patients with PAF and autoimmune and other non-synuclein diseases affecting the autonomic nervous system. P-syn was abundantly present in patients with PAF and found in 100% of skin biopsy samples from PAF and none of the samples from patients with autoimmune and other non-synuclein related pathology. Cutaneous p-syn is a promising minimally invasive early biomarker to confirm an underlying synucleinopathy, particularly in patients with atypical features, and may help avoid unnecessary trials of immune therapy and the associated costs and complications.

We then asked whether analysis of cutaneous p-syn could differentiate between patients with PAF and other α-synucleinopathies. Using a semi-quantitative score to assess the

distribution of cutaneous neural p-syn deposits, we found mean total p-syn scores at the distal leg were significantly higher in patients with PAF compared to patients with MSA and PD, in keeping with a peripherally predominant α-synucleinopathy.

We then investigated the relationship between p-syn deposits on autonomic nerves and quantitative autonomic testing. Mean autonomic p-syn score, reflecting p-syn deposits on autonomic adnexa, correlated with ΔSBP on tilt, OIR-tilt and PRT, quantitative autonomic biomarkers affected by adrenergic control of total peripheral resistance, suggesting p-syn deposition in peripheral autonomic nerves may impair autonomic control of peripheral vascular resistance leading to OH.

8.1.5 Cutaneous autonomic denervation correlates with cardiovascular autonomic failure in MSA and PD (Chapter 7)

Finally, we explored the relationship between OH, cardiovascular autonomic failure and cutaneous autonomic denervation in patients with MSA and PD with and without autonomic failure in a multicentre study. All patients were assessed with a core protocol of cardiovascular autonomic testing, and in addition, patients assessed at our centre had in-depth cardiovascular autonomic testing with beat-to-beat recordings of heart rate and blood pressure, and measurement of plasma noradrenaline.

We found that in patients with MSA and PD, OH was associated with evidence of more widespread sympathetic and parasympathetic autonomic failure, suggesting it occurs in the context of a more global impairment of autonomic reflexes rather than in isolation, as is found in some elderly patients without neurodegenerative diseases. The severity of OH as assessed by the fall in systolic blood pressure on standing correlated with cutaneous adrenergic denervation, patient reported autonomic symptoms, and clinical scales

assessing functional disability. Patients with MSA and OH had significantly higher supine noradrenaline levels than PD with OH patients, but there were no other significant differences when evaluating cardiovascular autonomic testing and cutaneous innervation. In MSA, the underlying pathophysiology of neurogenic OH has previously been thought to be predominantly through dysfunction of central autonomic networks, in contrast to dysfunction of peripheral postganglionic autonomic nerves in PD. Our study suggests postganglionic adrenergic denervation may contribute to the pathophysiology of OH in patients with MSA as well as patients with PD, with potential impact on responses to therapeutic agents. The findings on the core cardiovascular autonomic testing protocol correlated well with the more comprehensive protocol used in a subset of patients. If validated in larger cohorts, the core protocol shows promise as an abbreviated tool to screen for autonomic failure in patients with α-synucleinopathies.

8.2 Limitations

8.2.1 Multimodal autonomic function tests

Our multimodal approach capitalised on the expertise available within our centre in cardiovascular autonomic testing, sudomotor testing, neuro-ophthalmology, uro-neurology included quantitative objective autonomic testing and patient reported outcomes covering several autonomic domains. Our protocol did not objectively assess gastrointestinal function, which should be explored given the prominent gastrointestinal symptoms in some of the patient cohort.

In our studies, we found pupillometry was a valuable diagnostic and prognostic biomarker, helping to differentiate patients with gAChR-positive and gAChR-negative autoimmune autonomic failure, PAF and other α-synucleinopathies, as well as predicting

phenoconversion in patients with PAF. Some of our more disabled patients, including more elderly patients with neurodegenerative diseases, and those with severe orthostatic intolerance, were not able to tolerate an additional visit to our neuro-ophthalmology clinic to undergo formal pupillometry. We plan to validate our current protocol for assessing pupillary function on handheld pupillometers to enable bedside assessment of a wider patient group and to incorporate formal pupillometry as part of the routine assessment in all patients presenting to our centre with autonomic failure.

We used the DST to assess postganglionic sudomotor function, that was validated on a cohort of patients and normal controls from Italy, and will not fully represent the diverse ethnicities of a British population. Most of the large studies on sudomotor function worldwide to date have been performed in Western and predominantly Caucasian populations. Following the work from this PhD, we are planning a large multicentre study to increase the size and diversity of the normative dataset available.

Our group has recently optimised protocols for indirect immunofluorescence studies for cutaneous p-syn deposits and developed a simple and quick semi-quantitative score to assess cutaneous p-syn distribution. We plan to explore more automated methods of quantifying p-syn deposits described by other groups.[132, 133]

8.2.2 Retrospective studies

For our retrospective studies, we had the benefit of being able to obtain raw data on autonomic testing for relatively large numbers of patients with rare autoimmune and neurodegenerative patients seen at a national referral centre over twenty years. Some patients seen several years ago were deceased at the time these studies were conducted, and retrospective clinical information available from patient notes at times

more limited, but whenever possible, we clarified missing information through direct correspondence with local teams and primary care physicians. For each group of patients studied, we conducted prospective studies where patients were deeply characterised, with results from the prospective studies correlating well with findings from the retrospective studies.

8.2.3 Patient recruitment

We largely studied patients referred to a national autonomic referral centre, therefore introducing a referral bias where we typically included patients with severe disease and atypical presentations, with relatively few numbers of patients with more typical presentations of idiopathic PD. Amongst the patients studied with MSA and PD, the diagnoses were made based on international clinical consensus criteria, with about a third of the MSA cohort comprising of pathologically confirmed cases on post-mortem studies via the Queen Square Brain Bank. Previous clinicopathological series have shown a 62-79% rate of accuracy in clinical diagnoses of MSA vs post-mortem studies.[134, 135] Nevertheless, patients referred to our centre for specialist review were reviewed by clinicians with expertise in autonomics and movement disorders and followed up in our clinical service, often for several years, to monitor for the development of new symptoms and signs, with patients with unclear and alternative diagnoses excluded from final analyses.

8.2.4 Effects of COVID-19 pandemic

The prospective studies presented in this thesis were affected by the COVID-19 pandemic, which occurred in the second year of the PhD. Some planned clinical visits were converted to telephone reviews rather than in person, due to national restrictions,

which affected our ability to perform follow up testing and collect longitudinal biopsies in the time frames that were originally planned. There was reluctance amongst some clinicians and patients in commencing immune therapy in some gAChR-negative patients with more longstanding disease, resulting in fewer follow up skin biopsies from gAChR-negative patients collected to date. International restrictions during the COVID-19 pandemic also impacted on my visiting fellowship to Prof Maria Nolano's skin biopsy laboratory in Italy, which had to be delayed to the final year of the PhD. Prof Nolano's team and I therefore developed an abbreviated 3-well rather than standard 9-well protocol described in Chapter 2, that I used to process samples from some of the autoimmune autonomic failure patients, so that I was able to analyse more biopsies and still answer our key research questions in the time available.

8.3 Future directions

8.3.1 Multimodal biomarkers in autoimmune autonomic failure

We have described the largest cohort of patients with autoimmune autonomic failure studied with novel multimodal biomarkers before and after immune therapy. The use of objective biomarkers to quantify responses to immune therapy have informed management decisions for individual patients at our centre and incorporated to recent national guidelines on the use of intravenous immunoglobulin in patients with autoimmune autonomic ganglionopathy. Further longitudinal multimodal studies are ongoing, with plans to describe the longer-term outcomes in patients treated with immune therapy. In Chapter 3, we described a series of quantitative biomarkers to objectively monitor response to immune therapy in gAChR-positive patients, including OIR-tilt, Valsalva ratio, saliva production, and pupillary light reaction. In Chapter 4, we

showed, in contrast, the gAChR-negative patients had a heterogenous clinical phenotype, with a large proportion demonstrating preserved cholinergic function, which was consistently impaired in the gAChR-positive group. Our work suggests gAChR-negative patients should be fully assessed with a multimodal assessment of their autonomic and other neurological deficits at baseline to define their clinical phenotype and determine an individualised protocol for monitoring response to treatment. Amongst the gAChR-negative cohort, we identified patients with other antibodies and clinical investigations consistent with Sjogren's disease and GFAP-related autoimmune disease that responded to immune therapy. Previous studies have described autonomic symptoms in these cohorts but not described their objective autonomic testing in detail. We are planning to fully characterise the autonomic phenotype of these patient cohorts alongside insights from pathological samples.

8.3.2 Skin biopsies as an in-vivo method of studying postganglionic denervation and recovery

The longitudinal skin biopsies that we have studied to date have shown striking evidence of recovery and regeneration of somatic and autonomic nerve fibre populations in autoimmune autonomic patients both with and without the gAChR antibody. Punch skin biopsies are safe, well-tolerated, and far less invasive than biopsies of peripheral nerves, with repeat samples allowing us to study the natural history and response to treatment of somatic and autonomic nerve populations in different diseases. Our protocols could be adapted to study other inflammatory neuropathies like chronic inflammatory demyelinating neuropathy or paranodal neuropathies, where markers against myelin basic protein and paranodal structures can be used to study structural changes to

myelinated nerve fibre populations and paranodal and nodal architecture before and after treatment.

8.3.3 Multimodal biomarkers in α-synucleinopathies

My thesis also includes the largest international cohort of patients with α-synucleinopathies assessed with cardiovascular autonomic testing, plasma catecholamines, and pupillometry and followed up at a single national referral centre, allowing characterisation of the autonomic phenotype at first presentation and analysis of predictors for phenoconversion in patients with PAF (Chapter 5). Previous natural history studies of patients with PAF and other synucleinopathies have typically described findings of cardiovascular and sudomotor testing, with smaller numbers with plasma catecholamines, and did not systematically evaluate pupillary function. We now have a cohort of prospectively recruited, deeply phenotyped patients with novel biomarkers including pupillometry, bladder studies, and skin biopsies with quantification of somatic and autonomic innervation and neural p-syn deposits (Chapter 6). We intend to follow up this cohort longitudinally to understand if any of the novel biomarkers studied can help to predict phenoconversion in patients presenting with PAF. Studying the evolution of this cohort over time may help us to understand whether patients with PAF represent ‘forme fruste’ of the other α-synucleinopathies, or whether those who convert to MSA have distinct features from early on in their disease course, and should be labelled as prodromal MSA, as outlined in the most recent international criteria laid out by the Movement Disorders Society. The development of more robust early diagnostic biomarkers will facilitate recruitment to future clinical trials for these neurodegenerative

diseases, at a stage where disease modifying treatment is more likely to have a clinically beneficial effect.

8.3.4 Cutaneous p-syn as a diagnostic biomarker and widening the clinical spectrum of PAF

The use of objective biomarkers to monitor all patients undergoing trials of immunotherapy allowed us to identify patients without the gAChR antibody who were suspected to have possible autoimmune autonomic failure but did not have objective improvements in clinical testing, and subsequently developed features consistent with a neurodegenerative disease. These atypical cases emphasised the importance of monitoring both objective clinical outcomes and patient reported outcomes when administering trials of immunotherapy, and the need for early diagnostic biomarkers to confirm a pathological diagnosis of PAF. We found abundant neural p-syn deposits in skin biopsies from the patients in our prospective study with PAF (Chapter 6), including those with atypical features that led to trials of immune therapy. Estimates for costs of immunotherapy are in excess of £4000 for 2g/kg of IVIg and £1800 for a series of 5 plasma exchanges, giving a total of over £34,600 spent on four patients in our series, and it is likely that there are patients with atypical features undergoing similar trials in other centres worldwide. More widespread use of cutaneous p-syn as a diagnostic marker for PAF may avert unnecessary trials of immune therapy and the associated costs and complications. It is also likely to expand the clinical spectrum of cases that are recognised to be due to this peripherally predominant α-synucleinopathy.

8.4 Final conclusion

In summary, this thesis has deeply characterised rare and disabling autoimmune and neurodegenerative autonomic diseases, capitalising on the unique cohort of patients and multi-disciplinary specialist expertise available at the National Hospital for Neurology and Neurosurgery, as well as national and international collaborations. The use of objective quantitative biomarkers to improve the early diagnosis and monitoring of patients with autonomic diseases represents an important, clinically relevant advancement in the field of autonomic neurology, with influence on national guidelines for immunoglobulin treatment and leading to changes in practice at our centre and other centres worldwide. We have identified important insights about disease pathophysiology and recovery following treatment, and validated novel biomarkers which can be used to study other related diseases in the future.

9. References

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10. Author contribution statement

1. Cardiovascular autonomic function data and plasma catecholamines were measured by clinical autonomic scientists as part of routine clinical testing at the Autonomic Unit at the National Hospital for Neurology and Neurosurgery (all chapters).
2. I conducted the pupillometry, bladder studies and secretomotor testing for the prospective patients (Chapters 3, 4, and 5). Pupillometry for retrospective patients was performed by Mr Fion Bremner, Consultant Neuro-ophthalmologist at the National Hospital for Neurology and Neurosurgery as part of the clinical work up for these patients.
3. I performed and analysed the dynamic sweat testing (DST) on the autoimmune autonomic failure patients (Chapters 3 and 4). The patients with α-synucleinopathies had DST performed and analysed either by me or other clinical research fellows and scientists working on a multi-centre study on α-synucleinopathies (Chapters 5, 6, 7).
4. Skin biopsies for our illustrative case in Chapter 3 were processed and analysed by Prof Nolano's team at the Skin Biopsy laboratory at the Maugeri Institute in Telese Terme.
5. I performed the skin biopsy analysis and quantification of intraepidermal and pilomotor nerve fibres for patients with autoimmune autonomic failure (Chapter 4).
6. Skin biopsy samples for patients with α-synucleinopathies were processed by Prof Nolano's team. I then quantified the cutaneous innervation and deposits of

phosphorylated synuclein in the samples from the α-synucleinopathy, autoimmune and other non-synucleinopathy patients (Chapter 6).

7. For the multicentre study in MSA and PD in Chapter 7, cardiovascular and sudomotor testing at the Italian sites was performed by local clinical scientists. Skin biopsy analysis and quantification of intra-epidermal, sudomotor and pilomotor nerves was performed by Prof Nolano's team. I collated the data and performed the statistical analysis for this study.

11. Research output during PhD

11.1 First author publication on work directly related to PhD

1. Koay S, Vichayanrat E, Bremner F, et al. Multimodal Biomarkers Quantify Recovery in Autoimmune Autonomic Ganglionopathy. Ann Neurol 2021;89:753-768 (Appendix 1).[1]

11.2 Co-author publications on work performed during but not directly for PhD

1. Vichayanrat E, Valerio F, Koay S, et al. Diagnosing Premotor Multiple System Atrophy: Natural History and Autonomic Testing in an Autopsy Confirmed Cohort. Neurology 2022.[105]
2. Provitera V, Iodice V, Manganelli F, et al. Postganglionic Sudomotor Assessment in Early Stage of Multiple System Atrophy and Parkinson Disease: A Morpho-functional Study. Neurology 2022;98:e1282-e1291.[110]
3. Nolano M, Caporaso G, Manganelli F, et al. Phosphorylated alpha-Synuclein Deposits in Cutaneous Nerves of Early Parkinsonism. J Parkinsons Dis 2022;12:2453-2468.[48]

11.3 Oral presentations on work directly related to PhD

1. ‘Cardiovascular autonomic failure correlates with autonomic denervation and patient symptoms in alpha-synucleinopathies’, EAN Congress, Vienna, June 2022.
2. ‘Cardiovascular autonomic failure correlates with cutaneous autonomic denervation and patient symptoms in alpha-synucleinopathies’, ABN meeting, Harrogate, May 2022

3. ‘Double-edged Sword. Autoimmune autonomic ganglionopathy and Lambert-Eaton myasthenic syndrome.’ BPNS Spring Meeting, Manchester, Mar 2022.
4. ‘Ultimate Autonomic Challenge’, American Autonomic Society Virtual Meeting, November 2021
5. ‘Immune attack against ganglia: is post-ganglionic denervation reversible?’, BPNS Autumn Meeting, London, Sept 2021.
6. ‘Autonomic Biomarkers’, Centre of Neuromuscular Diseases (CNMD) Seminar Series, London, Mar 2021.
7. ‘Seropositive autoimmune autonomic ganglionopathy: clinical phenotype and autonomic biomarkers to monitor treatment response', EAN Virtual Meeting, May 2020.
8. ‘More than Holmes-Adie’, BPNS Autumn meeting, London, Nov 2018.
9. ‘Limited autoimmune autonomic ganglionopathy’,Autonomic Specialist Interest Group, ABN Autumn Meeting, London, Sep 2018.
10. ‘Autoimmune Autonomic Ganglionopathy: The National Hospital for Neurology and Neurosurgery/Queen Square Experience’. Scientific (oral) presentation at European Federation of Autonomic Societies Meeting, Vienna, Austria, July 2018.
11. ‘Autoimmune Autonomic Ganglionopathy: The NHNN Experience’. Platform presentation, ABN Annual Meeting, Birmingham, May 2018.

11.4 Poster presentations on work directly related to PhD

1. ‘Cutaneous phosphorylated synuclein as a diagnostic biomarker in potential pure autonomic failure’, American Autonomic Society Meeting, Hawaii, November 2022.
2. ‘Autonomic biomarkers and cutaneous phosphorylated α-synuclein deposition differentiate pure autonomic failure from other α-synucleinopathies’, American Autonomic Society Meeting, Hawaii, November 2022.
3. ‘Dynamic sweat testing: a novel method of assessing post-ganglionic sudomotor function in autoimmune autonomic ganglionopathy’. ABN Annual Meeting, Edinburgh, June 2019.

Appendix 1.

‘Multimodal Biomarkers Quantify Recovery in Autoimmune Autonomic Ganglionopathy.’

Research article published in Annals of Neurology.

Appendix 2.

Commissioning Criteria Policy for the use of therapeutic immunoglobulin (Ig) England, 2021.

Prepared by NHS England Immunoglobulin Expert Working Group.

Published by NHS England, in electronic format only.

Commissioning Criteria Policy for the use of therapeutic immunoglobulin (Ig) England, 2021

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Summary

The updated commissioning criteria for the use of therapeutic immunoglobulin (Ig) 2021 describes all conditions for which Ig is commissioned and provides the detail around the role, dose and place of Ig in the treatment pathway for individual indications alongside possible alternative treatment options for use of Ig in both adults and children. It has been built on a previous review of the literature updated with a further evidence review, expert opinion and multi-organisational input. The criteria have been developed by the Ig expert working group following wide consultation with specialty experts, relevant scientific societies and the respective Clinical Reference Groups (CRGs) for haematology, immunology, neurology, infectious diseases, rheumatology and other specialities. The CRG will review the document as per NHS England and NHS Improvement policy review process or when there is a significant change in evidence. Recommendations on Ig dose and outcomes are based on a combination of available evidence and expert opinion. The colour coding scheme, which had been previously devised for demand management but was often utilised as a commissioning tool, has now been replaced by categorisation of Ig use; to routinely commissioned or not commissioned routinely (NRC) categories. This is now based on the strength of clinical evidence.

Commissioning criteria

These commissioning criteria are for all indications previously categorised as red (conditions for which Ig treatment is considered the highest priority because of a risk to life without treatment) and blue (conditions for which there is a reasonable evidence base for the use of Ig but other treatment options are available) and those grey indications (immune-mediated disorders with limited or little/no evidence) that have moved into routine commissioning.

This guideline supersedes previous clinical guidelines and NHS England and NHS Improvement guidance with the exception of those indications within the Department of Health and Social Care (DHSC) 2011 clinical guidelines for immunoglobulin use[1], which have not moved into routine commissioning.

A completed referral form is still required for use of Ig in all indications. If the “Prior panel approval required” column states “No” - treatment can proceed without panel approval but a completed application form should be submitted and retrospectively reviewed by the Panel. If the column states “Yes”, treatment should not proceed without prior panel approval; if this is not possible, for example in an urgent case, retrospective

approval must be sought. For urgent approvals in hours – a process will need to be in place on the agreed pathway for approval. For those cases that require out of hours approval, panels will have local processes in place, to ensure robust governance for retrospective panel approval. Where local expertise is not available, panels will also be able to advise on dose optimisation and trials of treatment withdrawal.

All referrals should be carried out via the Medicine Database Solutions and Services (MDSAS) National Immunoglobulin Database e-referral platform. MDSAS data will be reviewed and findings reported to the Ig clinical expert working group and CRG with any recommendations on changes in policy will be updated in line with recommendations. MDSAS data will be analysed for ethnic groups to ensure any possible inequality in access is identified.

Indications or clinical scenarios not listed in "Commissioning Criteria Policy for the use of therapeutic immunoglobulin (Ig) England, 2021" are not routinely commissioned and will still require an Individual Funding Request (IFR) application subject to support by the Sub Regional Immunoglobulin Assessment Panels (SRIAPs), to be submitted to the national IFR Panel. If the IFR is approved, the diagnosis and locally agreed efficacy criteria are recorded on the immunoglobulin database.

In keeping with the advice included in previous iterations of these guidelines and to ensure cost-effective use and minimise dose-dependent adverse effects, Ig prescribing will be based on dose-determining weight (DDW), derived from ideal body weight (IBW)[2,3] using the following formula (available at: https://ivig.transfusionontario.org/dose). In a small minority of patients where this approach may be sub-optimal, higher doses of Ig may be required.

Vial Dosing

Total treatment course should be calculated and then rounded down to the nearest dose which can be administered using whole vials. Note in an adult patient part vials should never be used. Where the dose is split over multiple days, daily dose may differ.

For example:

- Male patient; Height 170cm; Weight 84kg
- Diagnosed with Guillain-Barre syndrome and meets criteria for IVIg, plan to receive 2g/kg based on DDW to be given over 5 days as per guidelines.

IBW = 50 + (0.91 x [Height(cm) – 152.4]) = 50 + (0.91 x[170-152.4]) = 66kg.
DDW = IBW + (0.4 x [ABW(kg) – IBW (kg)]) = 66 + (0.4 x [84-66]) = 73.2kg
Total dose = 2 x 73.2kg = 146.4g rounded down to nearest 5g (vial size) vial = 145g. To be split over 5 days suggested dosing:

Day 1: 30g Day 2: 30g Day 3: 30g Day 4: 30g Day 5: 25g

Ig and the use of IBW for dosing in paediatric patients

In all paediatric patients Ig dosing should be based on IBW. Actual body weight (ABW) should not be used. In patients whose ABW is < IBW, IBW should still be used to ensure appropriate dosing and preventing underdosing.

The recommended methods suggested by the RCPCH and NPPG to calculate IBW, include the use of the table at the back of the BNFc[4]or methods suggested in the UKMI document[5]. In the future, the RCPCH and NPPG aim to work on a standardised approach in conjunction with the BNFc.

Where possible doses should be rounded down to the nearest vial size to prevent wastage.

Use of Immunoglobulin in Immunology:

Immunoglobulin is routinely commissioned in the following indications, under the circumstances described:

Indications	Selection criteria	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose	Outcome measures to be recorded on the national database:	Prior panel approval required
HSCT in primary immunodeficiencies – long term use	PID patients undergoing HSCT	No	Ig is the only definitive treatment for antibody deficiency	Initiate at 0.4–0.6 g/kg/month; dosing requirements may increase and should be based on clinical outcome. Because of the possibility of B-cell reconstitution, evaluation of immune function (off Ig) is required at 2 years	• Trough IgG	No
Primary immunodeficiencies associated with significant antibody defects (excluding specific antibody deficiency) – long term use	A specific PID diagnosis must be established by a clinical immunologist In newly diagnosed patients with PID with no significant burden of infection, the decision to start Ig replacement should be based on a MDT discussion	No	Ig is the only definitive treatment for antibody deficiency	Initiate at 0.4–0.6 g/kg/month; dose requirements may increase and should be based on clinical outcome	• Trough IgG • Reduction in number of infections • Treatment courses of antibiotics • Days in hospital	No

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
Specific antibody deficiency – long term use	• Diagnosis by a clinical immunologist • Severe, persistent, opportunistic or recurrent bacterial infections despite continuous oral antibiotic therapy for 6 months • Documented failure of serum antibody response to unconjugated pneumococcal or other polysaccharide vaccine challenge	No, but see comments in column of position of immunoglobulin	Many patients with specific antibody deficiency will achieve protection from bacterial infections with prolonged antibiotic prophylaxis. Ig is reserved for those patients in whom antibiotic prophylaxis proves to be ineffective	Initiate trial at 0.4–0.6 g/kg/month for a period of 6 to 12 months; Long-term maintenance treatment should be based on clear evidence of benefit from this trial and require panel approval. Dose requirements may increase and should be based on clinical outcome	• Trough IgG • Reduction in number of infections • Treatment courses of antibiotics • Days in hospital • Database parameters will include entry of number of infections and days in hospital pre-treatment and 6 monthly thereafter.	Yes
Secondary antibody deficiency – long term use	• Underlying cause of hypogammaglobinaemia cannot be reversed or reversal is contraindicated. OR: • Hypogammaglobinaemia associated with drugs, therapeutic monoclonals targeted at B cells and plasma cells (rituximab and other anti-CD20, CD19 agents, daratumumab etc) post-HSCT*, NHL, CLL, MM or other relevant B-cell malignancy confirmed by haematologist AND • Recurrent or severe bacterial infection despite continuous oral antibiotic therapy for 6 months • IgG <4 g/L (excluding paraprotein) • Documented failure of serum antibody response to unconjugated pneumococcal or other polysaccharide vaccine challenge • It is recognised that vaccine challenge may be of limited value in patients with very low serum IgG (< 3g/L). In these circumstances vaccine challenge may	No, but see comments in column of position of immunoglobulin	Many patients with secondary antibody deficiency will achieve protection from bacterial infections with prolonged antibiotic prophylaxis. Ig is reserved for those patients in whom antibiotic prophylaxis proves to be ineffective Since infection susceptibility in patients with haematological malignancies is frequently multifactorial, the reduction in overall burden of infections with long term Ig replacement may be variable. For this reason, annual reviews of treatment are recommended. In patients with seasonal preponderance of infections, it may be appropriate to consider	0.4 – 0.6 g/kg/month modified to achieve an IgG trough level of at least the lower limit of the age-specific serum IgG reference range	• Trough IgG • Reduction in number of infections • Days in hospital • Database parameters will include entry of number of infections and days in hospital pre-treatment and 6 monthly thereafter. • Treatment courses of antibiotics	Yes

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
	be omitted if it is considered inappropriate clinically • It is acknowledged that not all of the above criteria will need to be fulfilled for an individual patient • In patients developing hypogammaglobinaemia associated with B-cell aplasia as a consequence of Chimeric Antigen Receptor – T cell therapy (CAR-T cells) targeted against B cell antigens, the prophylactic use of Ig in the absence of a burden of severe infections and vaccine challenge may be appropriate • Use of Ig post-CAR-T therapy in B-cell acute lymphoblastic leukaemia (B-ALL) Because of the severity of B-cell aplasia and the longer time required for reconstitution, it is anticipated that virtually all patients (children and adults) with B-ALL will initially require Ig replacement following CAR-T cell therapy. As with the use of Ig post-CAR-T therapy in B-cell lymphoma, continued use of IVIg should be reviewed at regular intervals based on B-cell recovery, serum immunoglobulins and burden of infection • Use of Ig post-CAR-T cell therapy in B-cell lymphoma The need for immunoglobulin replacement in patients receiving CAR-T cell therapy for B-cell lymphoma is variable ranging between 31% to 64% in published studies[6] highlighting faster B-cell recovery in this group in contrast to patients with B-cell acute lymphoblastic leukaemia		temporary cessation of Ig in the summer			

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
Thymoma with immunodeficiency – long term use	• Profound B cell depletion AND/OR • significant antibody deficiency	No	Ig is the only definitive treatment for antibody deficiency	Initiate at 0.4–0.6 g/kg/month; dose requirements may increase and should be based on clinical outcome	• Trough IgG • Reduction in number of infections • Treatment courses of antibiotics • Days in hospital.	No

* There is variable practice regarding Ig replacement in adult patients with hypogammaglobinaemia post-HSCT for haematological malignancy. The American Society for Blood and Marrow transplantation and the Canadian Blood and Marrow Transplant group have recently stated, “Don’t routinely give Ig replacement to adult HSCT recipients in the absence of recurrent infections regardless of the IgG level”[7].

It is possible that patients with recurrent sino-pulmonary infections on a background of chronic pulmonary GVHD and hypogammaglobinaemia may benefit if they fulfil the criteria for secondary antibody deficiency.

Use of Immunoglobulin in Haematology:

Immunoglobulin is routinely commissioned in the following indications, under the circumstances described:

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
Acquired red cell aplasia associated with chronic parvovirus B19 infection– short term use	Parvovirus B19 infection: • Parvovirus B19 infection confirmed by PCR, AND • Evidence of high viral load, usually above 109 IU/ml In cases of foetal hydrops: Likely to be associated with parvovirus B19	Infection other than parvovirus B19	Immunoglobulin is an adjunct to transfusion. Chronic parvovirus infection generally occurs on a background of immunosuppressive therapy, primary or HIV-related immunodeficiency and may resolve with a reduction in immunosuppression. Acute parvovirus infection associated with transient aplastic crisis requires urgent transfusion rather than Immunoglobulin	1.0 g/kg – 1.2g/kg in divided doses. This may be repeated on relapse and for a 2nd relapse	• Rise in haemoglobin • Transfusion independence • Reticulocyte count	Yes
Alloimmune thrombocytopenia (foetal-maternal/neonatal) (FMAIT NAIT)	<u>Prevention or treatment of foetal thrombocytopenia or haemorrhage:</u> • Clinical suspicion of FMAIT in the antenatal setting based on clinical and laboratory features: • Unexplained previous foetal death, haemorrhage, hydrocephalus or thrombocytopenia or known affected sibling, AND • The presence of maternal platelet-specific alloantibodies directed against current paternal antigens (most commonly HPA-1a or HPA-5b).	No	Maternal: Immunoglobulin is the primary treatment and sometimes combined with steroids Neonatal: First line treatment is with HPA-1a/5b – negative platelets which covers 95% of HPA incompatibilities	Maternal: The dose of IVIG and the gestation at which to start treatment should be tailored according to the history of NAIT in earlier pregnancies. A patient with a low-risk obstetric history (where the previous infant had thrombocytopenia but no intracranial haemorrhage) should be commenced on 0.5g-1.0/kg/week from 20 weeks' gestation. In high-risk pregnancies, treatment should commence from as early as 12 weeks' gestation with a dose of 1g/kg/week (where the previous fetus or neonate had	• Successful outcome of pregnancy i.e. no severe haemorrhage such as intracranial haemorrhage • Platelet count above $50x10^9$/L at time of delivery • Increment in neonatal platelet count	No – for NAIT Yes – for FMAIT

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
	<u>Prevention or treatment of neonatal thrombocytopenia or haemorrhage</u>: Clinical suspicion of NAIT in the neonatal setting based on clinical features suggestive of bleeding e.g. purpura and/or bruising and/or more serious bleeding and a low platelet count		responsible for NAIT. Platelet transfusion is effective immediately. In contrast, immunoglobulin is a second line treatment and works in approximately 75% of cases. It has a delayed effect over 24 – 48 hours. Immunoglobulin may be of value if there is prolonged thrombocytopenia with the aim of minimising the need for platelet transfusions	intracranial haemorrhage after 28 weeks' gestation), or 2g/kg/week (where the previous fetus or neonate had intracranial haemorrhage before 28 weeks)[8-12] Neonatal: 1g/kg; a 2nd dose may be required if thrombocytopenia persists		
Autoimmune haemolytic anaemia (AHA, including Evans syndrome) – short term use	AHA, including Evans syndrome: • Symptomatic or severe anaemia, except in patients with co-morbidities), AND • Refractory to conventional treatment with corticosteroids, OR • Corticosteroids contra-indicated, OR • As a temporising measure prior to splenectomy AHA in pregnancy: • Pregnant women with warm AHA refractory to corticosteroids OR with evidence of fetal anaemia. • Neonates of mothers with AHA who have evidence of haemolysis and rising bilirubin despite intensive phototherapy	No	Immunoglobulin is reserved for patients unresponsive to steroids or where steroids are contra-indicated	1-2g/kg in two to five divided doses. This may be repeated on relapse and for a 2nd relapse	• Rise in haemoglobin • Transfusion independence • Reduction in haemolysis markers (bilirubin, lactate dehydrogenase)	No – for treatment of acute episodes Yes – for repeat courses
Coagulation factor inhibitors (alloantibodies and	<u>Acquired von Willebrand disease (VWD)</u> • Life- or limb-threatening haemorrhage,	Acquired VWD associated with IgM monoclonal gammopathy	Immunoglobulin is a therapeutic option in acquired VWD, particularly in cases	Either 0.4g/kg for five days or 1g/Kg for two days	• Rise of factor level • Resolution of bleeding	Yes** **If prior approval is

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
autoantibodies) – short term use:	AND • Failure to respond to other treatments, AND/OR • Prior to invasive procedure Treatment directed by the haemophilia centre at which the patient is registered		associated with a IgG monoclonal gammopathy alongside other therapies – plasmapheresis, desmopressin, VWF-containing concentrates and recombinant Factor VII		• Number of bleeding episodes	not possible then treatment should proceed, and retrospective approval should be sought
Haemolytic disease of the newborn – short term use:	Adjunct to continuous multiple phototherapy in cases of Rhesus haemolytic disease, or ABO haemolytic disease: • Rising bilirubin despite intensive phototherapy (see NICE CG98[13]) • Prevention of foetal haemolytic disease in women with a previous history of this and confirmed red cell antibodies to current paternal or foetal antigens, to delay the need for intrauterine transfusions	No	Immunoglobulin is an adjunct to phototherapy Also see NICE CG98 guidance[13]	0.5g/kg over 4 hours	• Bilirubin level • Need for exchange transfusion • Long term morbidity	No
Haemophagocytic syndrome (Haemophagocytic lymphohistiocytosis or HLH) – short term use:	Diagnosis by a consultant haematologist or rheumatologist based on H-score* including: • pyrexia • organomegaly • multiple lineage cytopenias • triglycerides • fibrinogen • ferritin • serum aspartate aminotransferase • haemophagocytosis on bone marrow biopsy • long-term pharmacological immunosuppression * A score >169 is 93% sensitive and 86% specific for HLH)	No	Other therapies include IL-1 receptor inhibition (Anakinra) Please refer to NHS England policy[14]	2g/kg in two to five divided doses alongside corticosteroids (dexamethasone) as per HLH protocol. This may be repeated on relapse and for a 2nd relapse, where alternative therapies are not indicated or are contraindicated	• Improvement of cytopenias • Survival • Improvement of HLH markers – Ferritin/soluble CD25	Yes
Immune Thrombocytopenic	Immunoglobulin generally used in only 4 situations in ITP: 1) Life-threatening bleeding	No	Thrombopoietin mimetics may be useful substitutes in	Adults: 1g/kg as a single infusion.	• Increase in platelet count	No for acute ITP; the use of a 2nd dose

<table>
<tr><th>Indication</th><th>Eligibility criteria:</th><th>Exclusion criteria:</th><th>Position of immunoglobulin, taking into account alternative therapies:</th><th>Recommended dose:</th><th>Outcome measures to be recorded on the national database:</th><th>Prior panel approval required</th></tr>
<tr>
<td>Purpura (ITP) short term use:</td>
<td>2) Where an immediate increase in platelet count is required e.g. before emergency surgery or other procedure (see table for target platelet counts)
3) Where the patient is refractory to all other treatment to maintain the platelet count at a level to prevent haemorrhage. It may need to be given every 2-3 weeks during a period where other second line treatments are being tried
4) Moderate severity bleeding in patient at higher risk of subsequent severe bleed. Patients with mucosal bleeding or bleeding from multiple sites or a previous history of severe bleeding are at higher risk of a subsequent severe bleed

Bleeding severity as defined by the “Updated international consensus report on the investigation and management of primary immune thrombocytopenia 2019”[15]

Target platelet counts for surgery*
<table>
<tr><td>Procedure</td><td>Platelet count</td></tr>
<tr><td>Dentistry</td><td>>20</td></tr>
<tr><td>Simple dental extraction</td><td>>30</td></tr>
<tr><td>Complex dental extraction</td><td>>50</td></tr>
<tr><td>Regional dental block</td><td>>30</td></tr>
<tr><td>Minor surgery</td><td>>50</td></tr>
<tr><td>Major surgery</td><td>>80</td></tr>
<tr><td>Major neurosurgery</td><td>>100</td></tr>
</table>
ITP in pregnancy:
Maintenance treatment with Ig may be required antenatally to maintain platelets above 20x10^9/l and/or to increase platelets to over 50 x10^9/l for</td>
<td></td>
<td>some patients (in situation 3) or as an adjunct in the other situations</td>
<td>A 2nd dose may be required after 24 – 48 hours, if severe or life-threatening bleeding:
e.g. Intracranial bleed or pulmonary haemorrhage
Otherwise, if a haemostatically adequate platelet count is not achieved a 2nd dose (1g/kg) may be considered at day 5 to 7

<u>Children</u>: 0.8 – 1g/kg as a single infusion.
A 2nd dose may be required after 24 – 48 hours, if severe or life-threatening bleeding, such as an intracranial bleed or pulmonary haemorrhage.
Otherwise, if a haemostatically adequate platelet count is not achieved a 2nd dose (1g/kg) may be considered at day 5 to 7</td>
<td>• Resolution of bleeding
• Number of bleeding complications</td>
<td>should be discussed with the designated panel lead.

Yes – for maintenance treatment</td>
</tr>
</table>

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
	delivery in women with symptomatic persistent or chronic ITP where other treatments have failed *There is controversy regarding the target platelet count for epidural anaesthesia[16]. There are no data to support a minimum platelet count and each case must be carefully considered. In the absence of bruising, bleeding history, and anticoagulation and if the INR, APTT and fibrinogen levels are normal, a small consensus of obstetric anaesthetists agree no changes to normal practice are needed until the platelet count drops below 50.					
Covid Vaccine-induced thrombosis and thrombocytopenia (VITT)	Confirmed/Probable diagnosis of VITT made by a haematologist conforming to up to date guidance from the Expert Haematology Panel - See British Society for Haematology website for details. Also see NICE NG200 guideline[17].	Isolated thrombocytopenia or thrombosis: • Reduced platelet count without thrombosis with D dimer at or near normal and normal fibrinogen. • Thrombosis with normal platelet count and D dimer	Treatment with intravenous immunoglobulin, irrespective of the degree of thrombocytopenia is urgent as this is the treatment most likely to influence the disease process. A repeat course of IVIg may be required depending on clinical course	1g/kg (divided over two days if required)	• Platelet count	No
Post-transfusion hyperhaemolysis – short term use **Prevention of haemolysis in patients with a history of transfusion-**	Treatment of acute post-transfusion hyperhaemolysis: Symptomatic or severe anaemia (Hb <60g/L, with evidence of on-going intravascular haemolysis due to a delayed haemolytic transfusion/hyperhaemolysis). It is	No	In combination with steroids, Immunoglobulin is used as first-line treatment	2g/kg (usually over two days) given with IV methylprednisolone 1-2g/kg over two or five days given with steroids	• Rise in haemoglobin • Transfusion Independence • Reduction in haemolysis markers (bilirubin, lactate dehydrogenase) • No haemolysis • Maintenance of post-transfusion Hb at 1 – 3 weeks	No

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
associated hyperhaemolysis **Prevention of delayed haemolytic transfusion reaction**	recognised that some patients with an Hb > 60 g/l may require treatment. Patients who have had previous delayed haemolytic transfusion reactions/post-transfusion hyperhaemolysis or who have single or multiple allo-antibodies AND who may require a blood transfusion	Eculizumab is commissioned as a 2nd line treatment where 1st line treatment has failed; Rituximab is recommended as a 3rd line treatment[18].		1 – 2 g/kg over 2 to 5 days, given with IV methylprednisolone	• Avoidance of need for repeated transfusion	
Post-transfusion purpura – short term use:	• Sudden severe thrombocytopenia 5 to 10 days post-transfusion of blood products, AND • Active bleeding (typically occurs in Caucasian HPA-1a antigen negative females previously exposed to HPA-1a antigen in pregnancy or transfusion)	No	There are now very few cases in UK following the implementation of universal leucocyte-reduction of blood components in 1999	1 - 2g/kg in divided doses over two to five days	• Increase in platelet count • Resolution of bleeding • Number of bleeding complications	No

Use of Immunoglobulin in Neurology:

Immunoglobulin is routinely commissioned in the following indications, under the circumstances described:

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
Acute idiopathic/autoimmune dysautonomia/ganglionopathy	• Acute onset autonomic failure with presence of ganglionic (alpha3) acetylcholine receptor antibodies OR • Acute onset autonomic failure with clinical pattern consistent with above including pupillary involvement but without identifiable antibodies AND • Authorised by specialist autonomic unit	Non-immune causes of autonomic failure (for example primary autonomic failure (PAF) without pupillary involvement, MSA multisystem atrophy, diabetes mellitus	IVIG may be required to obtain rapid control, but may be substituted for by prednisolone, MMF, plasma exchange or other immunosuppressants which are preferable in the longer term	2g/kg over 5 days initially repeated at 6 weeks then titrated to optimal interval and minimum dose to achieve stability Annual reassessment with IVIG suspension as necessary	• Postural BP drop reduction with improved activities of daily living • Time to significant postural BP fall • Numbers of syncopal and pre-syncopal episodes • Oral dryness score • Diarrhoea and constipation frequency	Yes
Autoimmune encephalitides (AIE) (antibody associated)	• Non-infective encephalitis, with or without underlying teratoma or malignancy with known encephalitis associated antibody (e.g. LGI1, Caspr2, NMDAR, GAD, DPPX, AMPA, GABAb and others) AND • Functional disability caused by seizures, encephalopathy, stiffness, cognitive dysfunction or other relevant neurological sequelae	Infective encephalitis or other non-inflammatory cause of encephalopathy or seizures	Search for underlying malignancy and treat as appropriate Prednisolone/Methylprednisolone is first line, with or without Plasma Exchange (where this is available) Ongoing treatment with IVIG may be necessary where long-term oral immunosuppression, tumour removal and definitive strategies to reduce antibody levels (e.g. cyclophosphamide/rituximab) are ineffective or contra-indicated NB: Please note the Enceph-IG study is	2g/kg over 5 days initially repeated at 3 to 6 weeks. Repeat course 3 times if necessary. If repeated courses are required, consider institution of alternative longer-term strategy immediately	AIE outcomes for all types (except Ab titre in non-antibody associated) • Antibody titre (if relevant and measurable) • Modified Rankin Score • Seizure numbers • Improvement on one or more validated tests of memory or executive tasks • resolution of MR signal change (where present) • Resolution of hyponatraemia where present	Yes

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
			available[19]. Consider recruitment within the trial for suitable patients.			
Autoimmune encephalitides (no known antibody defined)	• Non-infective encephalitis, with or without underlying teratoma or malignancy without known encephalitis associated antibody AND • Functional disability caused by seizures, encephalopathy, stiffness, cognitive dysfunction or other relevant neurological sequelae AND • Evidence of inflammatory CNS disorder including active CSF, EEG defined seizures, MRI imaging changes consistent with AIE, known antibodies etc in the absence of infection	Infective encephalitis or other non-inflammatory cause of encephalopathy or seizures	Search for underlying malignancy and treat as appropriate. Prednisolone is first line, with or without Plasma Exchange (where this is available) Ongoing treatment with IVIG may be necessary where long-term oral immunosuppression, tumour removal and definitive strategies to reduce antibody levels (e.g. cyclophosphamide/ rituximab) are ineffective or contra-indicated NB: Please note the Enceph-IG study is available[19]. Consider recruitment within the trial for suitable patients.	2g/kg over 5 days initially repeated at 3 to 6 weeks. Repeat course 3 times if necessary If repeated courses are required, consider institution of alternative longer-term strategy immediately	AIE outcomes for all types • Modified Rankin Score • Seizure numbers • Improvement on one or more validated tests of memory or executive tasks • resolution of MR signal change (where present) • Resolution of hyponatraemia where present	Yes
CIDP (including IgG or IgA associated paraprotein associated demyelinating neuropathy)	• Probable or definite diagnosis of CIDP by a neurologist according to the EFNS/International Peripheral Nerve Society Guidelines. AND • Significant functional impairment inhibiting normal daily activities. All patients should have an initial documented assessment after induction dosing and a further assessment after 2-3 doses to demonstrate meaningful functional improvement. Annual withdrawal/clinical reviews should be performed to document on-going need.	No specific exclusion criteria but see general comments regarding prothrombotic risks of Ig	Ig should not always be considered first line treatment for CIDP, although it may be where steroids are contra-indicated and plasma exchange is not available. Where steroids, Ig and plasma exchange are all available Ig would be considered preferable in patients with motor predominant CIDP, rapidly progressive disease where rapid response is required (particularly patients requiring admission to hospital) or where steroids	An initiation regimen of a maximum 4g/kg divided into at least two courses of 1-2g/kg each, and given over a 4 to 8-week period, with assessment at the end of the period. Regimens to establish response might include: 2g/kg given over 2 to 5 days and repeated after 6 weeks[20]. 2g/kg initially followed by 1g/kg after 3 weeks and a further 1g/kg 3 weeks later[21].	Efficacy outcomes should be used to measure response after the chosen initial regimen and thereafter when assessing for dose optimisation Clinically meaningful improvement in any three of the following prespecified measures per patient: • MRC score (7 pairs of muscles in upper and lower limb scored 0–5, maximum 70) • INCAT sensory sum score • ONLS (Overall Neuropathy Limitation Score)	Short-term initiation treatment to assess Ig responsiveness – No Long-term treatment - Yes

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
			or plasma exchange are contra-indicated. Strong consideration should be given to the early use of steroids or plasma exchange in other circumstances	For maintenance dose optimisation see general note below	• Hand dynamometry • Inflammatory RODS score • 10-m walk (in seconds) • Up and go 10m walk (in seconds) • Berg Balance scale • Other validated disability score	
Guillain-Barre syndrome (GBS) (includes Bickerstaff's brain stem encephalitis and other GBS variants)	• Diagnosis of GBS (or variant) in hospital, AND • Significant disability (Hughes Grade 4). OR • Disease progression towards intubation and ventilation OR • mEGRIS score ≥ 3 OR • Poor prognosis mEGOS ≥ 4	Patients with mild and/or non-progressive disease not requiring intubation	Patients with Miller-Fisher Syndrome do not usually require IVIg and unless associated with GBS overlap with weakness will recover normally PLEX is equally efficacious as IVIg in GBS and should be preferentially considered where it is clinically appropriate and easily accessible	2g/kg as soon as possible after the diagnosis is confirmed, given over 5 days. Administration over a shorter time frame not recommended because of fluid and protein overload and pro-coagulant effects. IVIG is unlikely to be effective if given more than 4 weeks after the onset of symptoms[22]. Second doses of IVIg are not effective in the treatment of GBS and may be associated with real potential harm[23].	None	No
IgM Paraprotein-associated demyelinating neuropathy	• Diagnosis by a neurologist, AND • Significant functional impairment inhibiting normal daily activities. AND • Other therapies have failed, are contra-indicated or undesirable	Mild disease with non-progressive sensory loss and imbalance does not require treatment	IVIg is seldom significantly effective and response should be reviewed at least every 6 months if there is initial functional improvement. Alternative underlying haematological diagnoses should be considered which may direct treatment, or other therapies such as single agent rituximab (or biosimilars) should be considered. Rituximab is recommended in IgM paraproteinaemic demyelinating peripheral	An initiation regimen of a maximum 4g/kg divided into at least two courses of 1-2g/kg each, and given over a 4 to 8-week period, with assessment at the end of the period. Regimens to establish response might include: 2g/kg given over 2 to 5 days and repeated after 6 weeks[20]. 2g/kg initially followed by 1g/kg after 3 weeks and a further 1g/kg 3 weeks later[21].	Efficacy outcomes should be used to measure response after the chosen initial regimen and thereafter when assessing for dose optimisation Clinically meaningful improvement in any three of the following prespecified measures per patient: • MRC score (7 pairs of muscles in upper and lower limb scored 0–5, maximum 70) • INCAT sensory sum score • ONLS (Overall Neuropathy Limitation Score) • Hand dynamometry	Yes

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
			neuropathy in adults, in line with NHS England policy[24].	For maintenance dose optimisation see general note below	• Inflammatory RODS score • 10-m walk (in seconds) • Up and go 10m walk (in seconds) • Berg Balance scale • Other validated disability score	
Inflammatory Myopathies **Dermatomyositis (DM)** **Polymyositis (PM)**	• Diagnosis of myositis by a neurologist, rheumatologist, dermatologist or immunologist of DM or PM AND EITHER: • Patients with PM or DM who have significant muscle weakness; OR • Dysphagia and have not responded to corticosteroids and other immunosuppressive agents; OR • DM with refractory skin involvement.	No specific exclusion criteria but see general comments regarding prothrombotic risks of Ig	Where progression is not rapid and in the absence of contra-indications, steroids should be considered first. In adult patients (and post-pubescent children through the NHS England and NHS Improvement Medicines for Children policy[25]) with refractory disease associated with myositis-specific antibodies, rituximab (or biosimilar) has been approved as a second line treatment by NHS England[26]. Abatacept is recommended in refractory idiopathic inflammatory myopathies (adults and children aged 2 and over), in line with NHS England policy as a third line treatment[27]. IVIg would be the fourth line treatment line. IVIg is seldom effective in isolation and is best used as an adjunct to immunosuppressive therapy. Maintenance treatment with IVIg for a prolonged period (usually less than 12 months) may be required in a small minority of patients	An initiation course of a maximum 4g/kg divided into at least two courses of 1-2 g/kg each, and given over a 4 to 8-week period, with assessment after dosing. Regimens to establish response might include: 2g/kg given over 2 to 5 days and repeated after 6 weeks For maintenance dose optimisation see general note below The need for maintenance treatment in resistant juvenile dermatomyositis should be determined on an individual basis	Clinically meaningful improvement in three pre-defined measures from the list below: DM: functional/disability scores (ADLs): • semi-quantitative muscle scores (MRC sumscore) • other quantitative muscle strength (e.g. MMT8) • up and go 10-m walk (in secs) • CDASI • CAT or DAS • FVC • CHAQ to include the childhood score PM: functional/disability scores (ADLs): • semi-quantitative muscle scores (MRC sumscore) • other quantitative muscle strength (e.g. MMT8) • up and go 10-m walk (in secs) • HAQ • FVC Efficacy outcomes should be recorded after the initiation course and regularly reassessed and recorded thereafter For Dermatomyositis (juvenile – JDM): • MMT-8	Yes

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
			with inflammatory myositis, as a third line treatment after consideration of rituximab (see comments under position of immunoglobulin). In these cases, every effort should be made to establish the minimum clinically effective dose by either reduction of dose or lengthening the intervals between infusions. Cessation trials should be attempted at least annually to establish on-going need for treatment		• CMAS score • CK for baseline and asses how a patient has improved after each infusion or at least after 3 infusions. • PGALs is used to assess how many inflamed or swollen joints a patient has.	
Opsoclonus-myoclonus syndrome - paediatric or adult non paraneoplastic	• Paediatric OMS diagnosed by a paediatric neurologist OR • OMS in an adult with no evidence of neoplasm, anti-neuronal antibodies, or focal structural or inflammatory alterative diagnosis	Structural disease. Multiple sclerosis or other inflammatory lesions associated with defined diagnoses where the primary treatment of that disease is not Ig	Corticosteroids should be tried first Consider other anti-inflammatory strategies including oral immunosuppressants, rituximab or cyclophosphamide as appropriate	2g/kg over 5 days initially repeated at 6 weeks then titrated to optimal interval and minimum dose to achieve stability	• OMS score	Yes
Paraneoplastic neurological syndromes (PNS) without evidence of autoantibodies	• Defined paraneoplastic syndrome (for example limbic encephalitis, sensory ganglionopathy, cerebellar degeneration etc) AND • Evidence of a PNS associated tumour (e.g. small cell lung, ovarian or testicular, breast, thymoma etc	See eligibility criteria	Treatment of primary tumour Consider steroids and plasma exchange	2g/kg over 5 days initially repeated at 6 weeks. If beneficial then titrated to optimal interval and minimum dose to achieve stability. Discontinue If not objectively effective after 2 doses.	• Modified Rankin Scale • 10m walk Any validated relevant disability measure appropriate to the condition	Yes

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
Multifocal Motor Neuropathy (MMN)	• Diagnosis by a neurologist of multifocal motor neuropathy with or without persistent conduction block; AND • Significant functional impairment inhibiting normal daily activities	No specific exclusion criteria but see general comments regarding prothrombotic risks of Ig	No alternative treatments known	An initiation regimen of a maximum 4g/kg divided into at least two courses of 1-2g/kg each, and given over a 4 to 8 week period, with assessment at the end of the period. Regimens to establish response might include: 2g/kg given over 2 to 5 days and repeated after 6 weeks[20]. 2g/kg initially followed by 1g/kg after 3 weeks and a further 1g/kg 3 weeks later[21] For maintenance dose optimisation see general note below If no significant measurable and functionally meaningful improved in abilities has been achieved after 3 doses IVIg should be stopped	Improvement in 3 pre-specified measures from the below list: • MRC score • Power score from 7 pre-defined pairs of muscles including 4 most affected muscle groups neuro-physiologically • RODS for MMN • Hand dynamometry • ONLS • 10-m walk (in secs) • Any other validated MMN disability measure	Short-term treatment to assess Ig responsiveness – No Long-term treatment - Yes
Myasthenia Gravis (MG), includes Lambert-Eaton Myasthenic Syndrome (LEMs)	• Diagnosis of MG or LEMS by a neurologist AND EITHER. • Acute exacerbation (myasthenic crisis). OR • Weakness requires hospital admission. OR • Prior to surgery and/or thymectomy	No specific exclusion criteria but see general comments regarding prothrombotic risks of Ig	All patients requiring urgent in patient treatment should receive plasma exchange first if available, including considering transfer to an appropriate neuroscience centre. IVIg could follow plasma exchange if required Where plasma exchange is not available, IVIg may be appropriate In rare circumstances where a patient has failed all standard treatments (including steroids and immunosuppression) and where authorised by a specialist in MG from a	In acute exacerbation use plasma exchange first where available. Patients admitted to hospital should receive 1g/kg in the first instance, only receiving a further 1g/kg if there is further deterioration or no response. Patients with life threatening disease (ITU with respiratory and/ or bulbar failure) should receive 2g/kg	Improvement in variation of myasthenic muscular strength and fatigue measures by the QMGS MG composite score. Additional efficacy may be monitored using: • Forward arm abduction time (up to 5 min) • Quantitative Myasthenia Gravis Score (Duke) • Respiratory function, e.g. forced vital capacity • Variation of another myasthenic muscular score • Dysphagia score • Dysarthria 1-50 counting • Diplopia or ptosis measurement	Myasthenic crisis – No Long-term treatment - Yes

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
			centre with a specialist neuromuscular service, maintenance therapy may be considered A rituximab biosimilar agent is likely to be an equally effective alternative therapy and has been approved by NHS England[28] for this group of patients with resistant myasthenia	**Refer to dose optimisation section for maintenance**		
Neuromyotonia (Isaacs syndrome)	• Neuromyotonia from peripheral nerve hyperexcitablity associated with significant disability • AND • Supported by diagnostic electrophysiological changes with or without antibodies to the VGKCh complex (Caspr) and resistant to alternative agents	Non autoimmune myotonia syndromes	Anticonvulsants should be tried first from phenytoin, carbamazepine, sodium valproate and lamotrigine. Immunomodulation: Prednisolone +/- azathioprine or oral immunosuppressant Plasma exchange	2g/kg over 5 days initially repeated at 6 weeks then titrated to optimal interval and minimum dose to stability	• Timed up and go walk • Functional measure: e.g. Myotonia Behaviour Scale (MBS), Rivermead Mobility Index, or Brief Pain Inventory • Neurophysiological myotonia assessment	Yes
Non-MS CNS inflammatory disease covering the clinical phenotype of AQP4 ab disease, NMOSD, ADEM (with or without encephalopathy, including brainstem attacks), MOGAD, TM, ON **<u>Acute Disease: Short term use</u>**	• Acute disease attack* not responding to IVMP (5g-7g or equivalent in children) and PLEX. When PLEX is not available or delayed or contraindicated, IVIG can be used before PLEX	Mild relapses without: new neurological signs	Refractory to IV Methyl Prednisolone OR PLEX not available or contraindicated OR refractory to PLEX in cases of severe disability	2g/kg over 2-5 days	To be determined by disease features including 3 of: • Modified Rankin score • 10m walk • 9-hole peg test	No

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
	AND • Evidence of ongoing inflammation AND • Within 6 weeks unless evidence of active inflammation	OR reduced activities of daily living OR other inflammatory disease diagnoses (e.g. MS Sarcoid, Behcet's etc)	and ongoing inflammation (usually within 6 weeks)		• Validated neuropsychometric testing • Improvement of other relevant validated scale • Objective relevant imaging improvement If ON - clinical improvement of VA If TM - either 1. EDMUS OR 2. ASIA	
<u>Chronic relapse prevention:</u>						
MOGAD (Myelin Oligodendrocyte Glycoprotein Antibody Disease)	MOGAD - refractory to (relapse* breakthrough) at least two treatments; one must be prednisolone and an immunosuppressant (any of MMF/Rituximab/AZA/methotrexate) OR serious side effects with prednisolone (adequate dose and length of time)	Pseudorelapse OR MS (may have low positive MOGAbs)	Failed 2 first line therapies	1g/kg daily over 2 days then 1g/kg monthly for first year (titrate to 2g/kg if relapses occur despite on-going steroid and IVIg at 1g/kg) Annual reviews for dose optimisation	Suppression of further relapses* Treatment Failure – defined as objective evidence of true relapse* on treatment	Yes
AQP4 NMOSD	AQP4 NMOSD - Failed or intolerant to 3 or more 'usual treatments' resulting in relapse*, including at least prednisolone (unless severe prednisolone side effects from adequate dose and time) + immunosuppressant (aza/ritux/MMF/methotrexate /ciclosporin or tacrolimus /PLEX or new RCT treatment if available)	Pseudo relapse	As per selection criteria	1g/kg monthly for first year; if break through consider 2g/kg Review annually	Suppression of further relapses* Treatment Failure – defined as objective evidence of true relapse* on treatment	Yes
Ab negative phenotypes	Failed or intolerant to 3 or more 'usual treatments' resulting in relapse* including at least prednisolone (unless severe prednisolone side effects from adequate	Pseudo relapse OR Other inflammatory	As per selection criteria	1g/kg 2 then monthly for first year	Suppression of further relapses*	Yes

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
	dose and time) PLUS immunosuppressant (aza/ritux/MMF/methotrexate /ciclosporin or tacrolimus /PLEX or new RCT treatment if available)	disease diagnoses (e.g. MS Sarcoid, Behcets etc)		Review at one year try reducing interval /dose with alternative options	Failure – defined as objective evidence of true relapse* on treatment	

*Attack or Relapse is a new or extended neurological symptom with signs that reflects the anatomical location of the inflammatory lesion (note a minority of early MOGAD TM may be difficult to visualise) that is not a fluctuating residual symptom of an old lesion and that usually persists for at least one week. However, acute treatment should not be delayed. Contrast enhancement is present in the majority during the acute phase.

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
Rasmussen's Encephalitis	When other therapies (such as steroids) have failed	No specific exclusion criteria but see general comments regarding pro-thrombotic risks of Ig	Immunoglobulin is reserved for patients unresponsive to steroids and other therapies.	2g/kg given over 2-5 days and repeated monthly for three months for initial trial	Seizure frequency with expected reduction of 30% to continue therapy	Yes
Stiff person syndrome (SPS) or variant	Diagnosis of SPS or a variant (stiff limb, PERM, etc) by a consultant neurologist Supportive criteria: • Demonstration of auto-antibodies to GAD, DPPX, amphyphysin, gephyrin or other stiff person associated antibodies AND/OR • Continuous motor unit activity at rest on EMG testing in paraspinal or affected limb musculature	No specific exclusion criteria but see general comments regarding pro-thrombotic risks of Ig	Consider plasma exchange as initial treatment Rituximab is likely to be equally effective but is not commissioned for this indication	An initiation regimen of a maximum 4g/kg divided into at least two courses of 1-2g/kg each, and given over a 4 to 8 week period, with assessment at the end of the period. Regimens to establish response might include: 2g/kg given over 2 to 5 days and repeated after 6 weeks[20]. 2g/kg initially followed by 1g/kg after 3 weeks and a further 1g/kg 3 weeks later[21]. For maintenance dose optimisation see general note below. If no significant measurable and functionally meaningful	Report on at least two of the measures below: • Reduction in stiffness • Up and go 10-m walk (in secs) • BRIT score • Number of spasms per day • Validated measure of functional abilities	Yes

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
				improved in abilities had been achieved after 3 doses IVIG should be stopped		

For many disorders where rituximab is a potential longer-term alternative to IVIg, the speed of response should be considered in determining treatment choice. IVIg can provide more rapid but temporary control and is likely to be the preferred option in emergency situations where an immediate response is required, for example in dysphagia and/or difficulty in breathing in inflammatory myositis.

Dosing optimisation for maintenance – general notes:

An ongoing issue for diseases that require long-term immunoglobulin treatment is that once significant and functional responsiveness to intravenous immunoglobulin (IVIg) is demonstrated for a patient using standard immunomodulatory dosing, the 'maintenance' dosing required to maintain the therapeutic response is not well characterised. In this update, the dosing recommendations for some neurological indications include 'time to relapse' as the interval between doses. This approach is supported by recent evidence from The Oxford Programme for Immunomodulatory Immunoglobulin Therapy, which was set up to review multifocal motor neuropathy (MMN) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) treatment with immunoglobulin. In view of the uncertainty of both remission and disease progression in CIDP and MMN, The Oxford Programme reviewed the dose and infusion frequency of patients on a regular basis and showed that increasing the infusion interval proved successful in some patients and resulted in treatment discontinuation[29].

An alternative approach based on establishing the 'time to relapse' following the first or second dose followed by dose reduction has also been proposed and is equally feasible[20]. This ensures patients who need no more than 1 or 2 doses are not exposed to unnecessary doses and those with ongoing needs are optimised to a minimal dose.

Based on evidence from randomised trials, it is likely that up to 40% of patients with CIDP may be able to discontinue treatment[30] after 6-12 months, although a significant proportion may relapse and require retreatment. For this reason, periodic trials of cessation of treatment are recommended, especially in patients who appear to be stable even if optimally treated. The demonstration of continued IVIG requirement by forced suspension on more than 2 or 3 occasions over a 5-year period probably indicates ongoing long term dependence and further withdrawals are highly unlikely to be effective. Referral to a specialist neurology centre is recommended as early as possible.

In inflammatory myositis, maintenance treatment with IVIg for a prolonged period (usually less than 12 months) may be required in a small minority of patients. In these cases, every effort should be made to establish the minimum clinically effective dose by either reduction of dose or lengthening the intervals between infusions. Cessation trials should be attempted at least annually to establish on-going need for treatment[31].

Specific exclusion criteria against the use of immunoglobulin have not been listed, but it is important to carry out benefit-risk analyses in certain patient groups: patients at high risk of thromboembolism (hypertension, diabetes, smoking, hypercoagulable states) should be counselled regarding the prothrombotic risks of immunoglobulin.

IgA deficiency is no longer considered a contra-indication to the use of immunoglobulin and should not be withheld because of theoretical concerns of adverse reactions. The role of anti-IgA antibodies in causing reactions is controversial and measurement of anti-IgA antibodies prior to undertaking treatment is not warranted.

Use of Immunoglobulin in Infectious Diseases:

Immunoglobulin is routinely commissioned in the following indications, under the circumstances described:

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
Hepatitis A	Immunoglobulin is recommended in addition to hepatitis A vaccine for contacts of hepatitis A who are less able to respond to vaccine • (those aged 60 or over, OR • those with immunosuppression and those with a CD4 count <200 cell per microlitre), OR • those at risk of severe complications (those with chronic liver disease including chronic hepatitis B or C infection)	See eligibility criteria	Hepatitis A vaccine is recommended in addition to immunoglobulin Vaccine should be administered within 2 weeks of exposure	Subgam: <10 years 500mg >10 years 1000mg To be given by intramuscular injection*. Given with vaccine in those at high risk, within 2 weeks of exposure (those over 60 years, immunosuppression, CD4 count <200 cell per microliter) and those at risk of severe complications. For those exposed between 2-4 weeks ago, immunoglobulin may also be offered to modify disease in those at risk of severe complications (i.e. chronic liver disease including chronic hepatitis B or C infection).	Outcome measures not routinely recorded on surveillance databases Immunoglobulin is issued nationally and locally; records are held of who immunoglobulin was issued for with respect to exposure to the hepatitis A virus.	Prior approval is via discussion with UKHSA health protection team* *Find your local protection team here: https://www.gov.uk/health-protection-team

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
Measles (immune-suppressed individuals)	Immunosuppressed individuals (Group A and Group B based on level of immunosuppression[32]) who have had a significant exposure to measles and are known to be susceptible (based on vaccine history and /or IgG testing).	See eligibility criteria	For immunosuppressed contacts IVIg is mainstay management	0.15g/kg of IVIg recommended ideally within 72 hours of exposure although can be given up to 6 days. Where exposure recognised late or found to be antibody negative between 6 and 18 days after exposure, IVIg may be considered following discussion with specialist clinician.	Prevention of measles	Prior approval is via discussion with UKHSA health protection team* *Find your local protection team here: https://www.gov.uk/health-protection-team
Measles (pregnant women and infants)	Pregnant women who have identified as susceptible based on vaccine history and /or antibody testing who have had a significant exposure to measles. Infants under 9 months of age with a significant exposure to measles. Advice is available at: https://www.gov.uk/government/publications/measles-post-exposure-prophylaxis	See eligibility criteria	For pregnant contacts, immunoglobulin is mainstay management for PEP For infants below 6 months immunoglobulin is mainstay treatment; For infants aged between 6-8 months, MMR vaccine can be offered if exposure occurred outside household setting AND ideally should be given within 72 hours	• For pregnant contacts, approximately 3000mg of human normal immunoglobulin (HNIG) • Infants 0.6ml/kg up to a maximum of 1000mg of HNIG HNIG to be given within 6 days of exposure in pregnant women and infants.	Prevention of measles	Prior approval is via discussion with UKHSA health protection team* *Find your local protection team here: https://www.gov.uk/health-protection-team

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
Polio	To prevent or attenuate an attack: • An immunocompromised person inadvertently given live polio vaccine, OR • An immunocompromised person whose contacts are inadvertently given live polio vaccine	See eligibility criteria	Immunoglobulin represents first –line treatment	<1 year: 250mg 1 – 2 years: 500mg >3 years: 750mg Stool samples from the immunosuppressed individual must be obtained one week apart. If poliovirus is grown from either sample, repeat immunoglobulin at 3 weeks. Continue weekly stool collection and administration of immunoglobulin three weekly until immunocompromised individual's stool is negative for poliovirus on two occasions	Either: • Prevention of infection, or • Resolution of infection	Prior approval is via discussion with UKHSA health protection team* *Find your local protection team here: https://www.gov.uk/health-protection-team
Severe or recurrent Clostridium difficile infection (CDI) colitis - short term use	• Severe cases (WCC >15 and/or, acutely rising creatinine and/or signs/symptoms of colitis) not responding to routine 1st line vancomycin and metronidazole • If multiple recurrences, especially with evidence of malnutrition	See comments under position of Ig	For fulminant or recurrent CDI unresponsive to appropriate antibiotics (see under selection criteria) consider IV tigecycline or IVIg[33]. Faecal microbiota transplantation is approved by NICE for patients with recurrent CDI unresponsive to antibiotics and is likely to be an effective alternative[34].	0.4 g/kg, one dose, and consider repeating once	• Clearance of C. diff. • Duration of hospital in-patient stay	Yes
Staphylococcal (including PVL-associated sepsis) or streptococcal toxic shock syndrome (TSS) - short term use	• Diagnosis of streptococcal or staphylococcal TSS, preferably with isolation of organism, AND	See comments under position of Ig	IVIg is reserved for patients with life-threatening disease who fail to achieve rapid improvement with antibiotic therapy. However, for streptococcal TSS, it should be noted that	Total dose of 2g/kg, because of uncertainty regarding the timing and optimal dose of IVIg, it is recommended that patients are reviewed after an initial dose of	• Improvement of FBC, ALK, CPK,and acute phase markers • Reduction in hospital inpatient stay • Survival	No Ideally, prior approval is recommended but if this is

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
	• Failure to achieve rapid improvement with antibiotic therapy and other supportive measures, AND • Life-threatening		there has been significant controversy regarding the benefits of IVIg treatment prompting the Infectious Diseases Society of America (IDSA) not to recommend its use in patients with necrotising Group A streptococcal infections[35] Since then a systematic review and meta-analysis of IVIg in clindamycin-treated patients with streptococcal TSS suggests a reduction in mortality from 33.7% to 15.7%, though this finding may be confounded by differences in baseline characteristics between patients receiving IVIg and those who didn't[36] Based on the results of this meta-analysis, the use of IVIg as adjunctive therapy is supported by Stevens DL[37].	1g/kg. Should there be no evidence of improvement at 24 hours, a further 1g/kg may be considered.		not possible, treatment should proceed, and retrospective approval should be sought.
Suspected tetanus case (IVIg)	Person with clinical symptoms suggestive of localised or generalised tetanus ("in the absence of a more likely diagnosis, an acute illness with muscle spasms or hypertonia AND diagnosis of tetanus by a health care provider")	See eligibility criteria	• Wound debridement • Antimicrobials • IVIG based on weight • Supportive care Vaccination with tetanus toxoid following recovery	Dosage based on equivalent dose of anti-tetanus antibodies of 5000 IU for individuals < 50kg and 10000 IU for individuals > 50kg See table below*	Resolution of tetanus infection	No
Tetanus prone injury (prophylaxis) (IM-TIg or SCIg)	Tetanus specific immunoglobulin (TIG) has limited stock and is recommended for susceptible individuals sustaining high risk tetanus prone injuries as defined in guidance[38].	See eligibility criteria	• Thorough cleaning of wound essential • Immunoglobulin for Prophylaxis • Booster of tetanus-containing vaccine for long term protection	<u>TIG</u>: • 250 IU for most uses • 500 IU if more than 24 hours have elapsed or there is a risk of heavy contamination or following burns The dose is the same for adults and children	Prevention of tetanus infection	No

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
				Immunoglobulin: If TIG (for intramuscular use) cannot be sourced, immunoglobulin for subcutaneous or intra-muscular use may be given as an alternative. Based on testing for the presence of anti-tetanus antibodies of alternative immunoglobulin products, the volume required to achieved the recommended dose of 250IU are included Although no time frame is specified in the guidance, IM TIG /immunoglobulin following a tetanus prone wound is only likely to confer benefit when given within incubation period of tetanus (10-21 days)		

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
Varicella zoster	Individuals for whom intra-muscular injections are contra-indicated (e.g. those with bleeding disorders) and thus cannot receive prophylaxis with VZIG IVIg is indicated for these Individuals who fulfil all of the following three criteria:	Mildly immunocompromised whose level of immunosuppression does not meet the criteria for	For those patients fulfilling eligibility criteria, there are no alternatives to IVIg	0.2g IVIG per kg body weight (i.e. 4ml/kg for a 5% solution) Brands have not been specified as no formal testing of products has been undertaken	Prevention of chicken pox infection Prevention of severe chicken pox	Prior approval is via discussion with UKHSA health protection team*.

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
	1) Significant exposure to chickenpox (varicella) or shingles (zoster) during the infectious period 2) At increased risk of severe chickenpox i.e. immunosuppressed individuals, neonates and pregnant women 3) No antibodies to varicella-zoster virus (based on VZV antibody testing) Immunosuppressed individuals are assessed at time of exposure into Group A & Group B based on likely level of immunosuppression Restrictions on use of VZIG have been in place since August 2018. Updated guidance on post exposure prophylaxis have been published in June 2019. Advice is available at: https://www.gov.uk/government/publications/varicella-zoster-immunoglobulin	either Group A or Group B do not require VZIG e.g. children on doses of prednisolone less than 2mg/kg/day, patients on doses of methotrexate 25mg/week or less A further dose of IVIg is not required if a new exposure occurs within 3 weeks of administration of VZIG or IVIG		VZIG (or IVIg when VZIG contraindicated) should be administered ideally within 7 days of exposure in susceptible immunosuppressed individuals. Where the exposure has been identified beyond 7 days, VZIG can be offered up to 14 days after exposure *Beyond this time for patients in both groups A and B, a discussion with the specialist caring for the individual should take place and IVIg (0.2g per kg body weight) may be considered in susceptible individuals for up to 21 days to attenuate infection*		*Find your local protection team here: https://www.gov.uk/health-protection-team
Viral pneumonitis post-transplantation: HSCT and solid organ	Definitive diagnosis of viral pneumonitis – Varicella Zoster Virus (VZV), Respiratory Syncytial Virus (RSV), Human Parainfluenza Virus (HPIV)	VZV - See comments under position of Ig RSV, HPIV – patients with mild disease confined to the upper respiratory tract	VZV - IVIg is reserved for patients with disseminated disease. For guidance on treatment of patients with significant exposure to chicken pox or herpes zoster please see use of Ig in specific infectious diseases. RSV, HPIV – patients with lower respiratory infections. In patients with RSV infection, Ig would be used as an adjunct to Ribavirin. For patients with RSV and HPIV upper respiratory infections post-HSCT, consider Ig in the presence of	1 – 2g/kg in divided doses	• Radiological improvement • Length of hospital stay • Survival	Yes. If prior approval is not possible then treatment should proceed, and retrospective approval should be sought.

Indication	Eligibility criteria:	Exclusion criteria:	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose:	Outcome measures to be recorded on the national database:	Prior panel approval required
			some or all of the following risk factors[39]: • Older age • GVHD • Lymphopenia : < 0.2 x 109/L • Neutropenia • Mismatched/unrelated donor • Immediate aftermath of HSCT (< 1 month)			

* Please note SPC currently indicates subcutaneous route of administration only (although previously indicate both s/c and im routes), PHE guidance recommends intramuscular administration for post exposure prophylaxis with Subgam.

*Dose of immunoglobulin in suspected tetanus cases.

IVIg Products tested for anti-tetanus antibodies	Volume required (in ml)	
	For individuals < 50kg	For individuals > 50kg
Gammaplex 5%, Intratect 5%, Flebogamma 5%, Vigam 5%, Octagam 5%	400ml	800ml
Privigen 10%, Octagam 10%, Intratect 10%, Flebogamma 10%, Panzyga 10%, Gammunex 10%	200ml	400ml

Use of immunoglobulin in "other" indications:

Indication	Eligibility criteria	Exclusion criteria	Position of immunoglobulin, taking into account alternative therapies:	Recommended dose	Outcome measures to be recorded on the national database:	Prior Panel Approval Required
Allo-immune neonatal haemochromatosis or gestational allo-immune liver disease (GALD)	• Pregnant mothers with a previous adverse pregnancy outcome and clear post-mortem evidence of fetal haemochromatosis or, • Women who have had an offspring with neonatal liver failure confirmed to be allo-immune neonatal haemochromatosis Decision to treat with Ig made by a consultant obstetrician with input from a liver unit specialist	No	For those patients fulfilling eligibility criteria, there are no alternatives to IVIg	Immunoglobulin is administered by intravenous infusion at a dose of 1g/kg (dose capped at 60g per week) to at risk mothers at 14 weeks, 16 weeks and then weekly from 18 weeks gestation until delivery between 37 and 38 weeks. The weight used to calculate the dose will be the mother's weight at booking	• Fetal loss (including gestation) • Gestation at delivery • Neonatal outcomes	Yes For further information please refer to the Clinical Commissioning Policy: Maternal intravenous immunoglobulin (IVIg) for the prevention of allo-immune fetal and neonatal haemochromatosis[40].
ANCA-associated systemic vasculitides (AAV)	• Patients with refractory/relapsing AAV in whom conventional immunosuppressive therapy is contra-indicated e.g. presence of severe infection or in pregnancy as bridging therapy • The role of IVIg in the treatment of ANCA negative small vessel vasculitis is unclear and each case will need to be assessed on individual grounds.	No specific exclusion criteria – see comments under selection criteria	IVIg is reserved as adjunctive or very rarely as sole therapy for the minority of patients in whom conventional immunosuppressive therapy is contra-indicated	Total dose of 2g/kg over 2 – 5 days every 4 weeks. The optimal duration of therapy is not known though most patients are likely to achieve remission after 3 months. IVIg should be discontinued after 3 months in the absence of clinical improvement.	• Improvement in Birmingham Vasculitis Score (BVAS)/PVAS to capture paediatric assessment tool • Fall in inflammatory markers • Improvement in organ function	Yes - Treatment cannot proceed without prior panel approval

Autoimmune uveitis - short term use	Severe aggressive sight-threatening disease unresponsive to conventional immunosuppressive treatment (topical and systemic steroids and oral or injectable immunosuppressants)	See comments under position of Ig	IVIg is reserved for exceptional cases where anti-TNF agents are contra-indicated or ineffective or associated with intolerable adverse effects and other corticosteroid and immunosuppressive agents are ineffective. Anti-TNF agents (Infliximab, Adalimumab) are regarded as the treatment of choice for the treatment of severe, refractory uveitis and are approved by NHS England[41])	1 - 1.5 g/kg/month – two to three infusions given 6 – 8 weeks apart to assess benefit	• Improvement or stabilisation in visual acuity • Imaging endpoints • Electrodiagnostic studies	Yes
Catastrophic antiphospolipid syndrome (CAPS)	Diagnosis of definite or probable CAPS: ▪ Thromboses in 3 or more organs developing in less than a week ▪ Histological evidence of microthrombosis in at least one organ ▪ Persistent anti-phospholipid antibody positivity (lupus anti-coagulant and or anti-cardiolipin/anti-B2GPI of IgG or IgM isotype	Chronic recurrent thrombosis due to other causes Thrombosis associated with stable anti-phospholipid syndrome in the context of other disorders	Steroids, anti-coagulants and plasma exchange (PLEX) represents optimal therapy IVIg is likely to be beneficial In selected cases associated with severe thrombocytopenia where PLEX is either unavailable or contra-indicated or in the event of deterioration following PLEX	2g/kg over 4-5 days	• Clinical improvement • Reduction in anti-phospholipid antibody levels	Yes - Treatment cannot proceed without prior panel approval
Immunobullous diseases - long term use	• Severely affected AND • conventional corticosteroid treatment with adjuvant immunosuppressive agents has failed or is inappropriate	See comments under position of Ig	IVIg is reserved as adjunctive therapy for patients with severe disease refractory to conventional immunosuppressive therapy. Rituximab is increasingly supplanting IVIg as the preferred treatment for resistant disease and is approved by NHS England[42] .In such patients it is listed as a 3rd line treatment	1 - 2 g/kg over 2–5 days. There may be a need for maintenance Ig in exceptional patients unresponsive or intolerant of Rituximab. In such cases every attempt should be made to define the minimal effective dose of Ig by undertaking periodic dose reduction and or lengthening the interval between treatment	• Reduction in recurrence of disease/relapse • Dose reduction/discontinuation of other immunosuppressive therapy • Improved quality of life • Resolution of blisters/healing affected skin • Resolution of pruritus	Yes

			alongside IVIg. However, Rituximab should be favoured over IVIg, given the stronger evidence base supporting its use			
Kawasaki disease – short term use **Paediatric inflammatory multisystem syndrome temporally associated to COVID-19 (PIMS-TS)**	Clinical diagnosis of Kawasaki disease by a paediatrician, paediatric infectious disease consultant or paediatric immunologist Clinical diagnosis of PIMS-TS by a paediatrician, paediatric consultant in infection or paediatric immunologist Clinical diagnosis of PIMS-TS in an adult (also known as MIS-A or AIMS-TS) by a consultant in infection or immunologist or appropriate specialist MDT" Because of the similarities between PIMS and Kawasaki disease, the use of IVIg is approved for any child fulfilling diagnostic criteria for PIMShttps://www.rcpch.ac.uk/	No	IVIg in combination with anti-inflammatory doses of Aspirin is the treatment of choice	2g/kg single dose, in conjunction with high-dose aspirin; a second dose may be given if no response, or if relapse within 48h	• Resolution of fever • Improvement in acute phase markers	No
Prevention of autoimmune congenital heart block (anti-Ro)	Prophylactic IVIg therapy has previously been given during pregnancy when: • There is a history of autoimmune congenital heart block in at least one previous pregnancy, AND	See comments under position of Ig	Hydroxychloroquine is regarded as the treatment of choice IVIg may be considered in exceptional cases refractory to hydroxychloroquine or if the patient is unable to tolerate hydroxychloroquine, but	Two infusions of 1g/kg/day, the first at 14 weeks and the second at 18 weeks of gestation	• Improvement in the degree of heart block at birth	Yes

	• Maternal anti-Ro and/or anti-La antibodies are present. However, more recent evidence has cast doubt on the beneficial effects of IVIg with hydroxychloroquine being regarded as first line therapy – see comments under position of Immunoglobulin		there is uncertainty regarding its efficacy. At a dose of 0.4 g/kg every 3 weeks administered from weeks 12 through to week 24 of gestation, IVIg was ineffective in preventing the development of CHB in neonates in two prospective open-label trials based on a case series a higher dose (1g/kg) alongside high dose oral prednisolone may possibly be effective.			
Transplantation (solid organ) – short term use	Antibody Incompatible Transplant (AIT): Patients in whom renal, heart, liver or lung transplant is prevented because of antibodies Antibody Mediated Rejection (AMR): Patients experiencing steroid resistant rejection or where other therapies are contraindicated after renal, heart, liver and/or lung transplant	See comments under position of Ig	While IVIg is included in many protocols, there is a paucity of high-quality evidence to support its use. A systematic review of AMR in kidney transplant recipients categorised the evidence supporting the use of IVIg as being 'very low'[43]. Where IVIg is used in combination with plasma exchange (PLEX), any beneficial effects of Ig are likely to be negated by subsequent PLEX. For this reason, the use of Ig immediately prior to PLEX is not supported. The addition of Rituximab to IVIg appears to be of benefit in lowering HLA antibody titres	AIT: Up to 2 g/kg to be repeated as per DSA; in renal desensitisation at 0.1 g/kg for 8–12 doses AMR: Treatment protocols vary in the UK ranging from low dose 100mg/kg after PLEX or high dose 2g/kg	AIT and AMR: Renal: • Type of renal transplant • HLA class DSA (where available) • Rejection episodes • Patient survival • Graft survival • Renal function = eGFR (MDRD) • Cardiothoracic: • DSA • Length of ITU and hospital stay Graft function (heart = rejection fraction; lung = spirometry; liver = liver function, clotting indices)	No

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Additional information

IFR form can be found at: https://www.england.nhs.uk/publication/specialised-services-individual-funding-requests/

More information on IFRs in general, including the application form, is available here: https://www.england.nhs.uk/commissioning/spec-services/key-docs/#ifr

Clinical Guidelines for Immunoglobulin Use (2nd edition update; July 2011):
https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/216671/dh_131107.pdf

NHS England will monitor use of Ig in grey indications via the Ig database and provide SRIAPs and commissioners with data relating to use in grey indications.